Causes of spatial variation in parasite and pathogen pressure in insects

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Philosophy

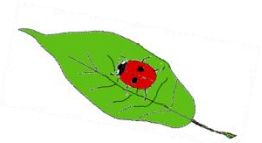
By Daria Pastok

May 2015

Abstract

The reproduction of the two-spot ladybird, *Adalia bipunctata*, is inhibited by a sexually transmitted ectoparasitic mite *Coccipolipus hippodamiae* that sterilizes female hosts, and a range of heritable microbes that kill male hosts during embryogenesis for instance *Spiroplasma*. Historical sampling indicated these two parasites were present commonly in *A. bipunctata* in the south of Sweden but were absent or rare in northern populations. In this thesis, I first established that the distribution of the mite was still stable over time, with a boundary to mite presence at 61˚N, as found 10 years previously. The incidence of male-killing *Spiroplasma*, in contrast, showed a small northward shift in its presence. I then examined the causes of mite presence/absence, and concluded ladybirds from northern populations were competent to carry and transmit mite infection, but that host phenology prevented its persistence in natural populations. I then explored the hypothesis that mite presence might select for increased reluctance of females to mate by comparing mating behaviour between mite present/mite absent populations. I observed that whilst rejection behaviour was protective against mite transmission, it was not more commonly observed in ladybirds derived from mite present populations. Finally, I examined whether the presence of male-killing *Spiroplasma* might affect mite epidemiology, and whether the mite itself might impact on the dynamics of the male-killing *Spiroplasma*. I observed that *Spiroplasma* did not affect individual competence to acquire and transmit mite infection, and did not protect its host against mite induced sterility. However, *Spiroplasma* was observed to mildly prolong host longevity, which may enhance the capacity of the mite to pass from overwintered to new generation cohorts of its host. Further, population sex ratio biases induced by *Spiroplasma* were predicted to influence mite epidemiology, through altering mating rate and per contact mite transmission probability. I therefore conclude first that mite incidence can be explained through host phenological variation, and that sex ratio distorting symbionts may impact on the dynamics of the mite infection. Future studies should explore the causes of high prevalence *Spiroplasma* infection in *A. bipunctata* in southern Sweden, and the features that prevent the establishment of infection in the north of the country.

Acknowledgements



PhD has been the most challenging time in my life. It has been an also amazing time. I have met a lot of wonderful people who gave me a lot of support, who motivated me and made me laugh. I would like to thank all of them. Without their support I wouldn't be here where I am at the moment. I don't want to miss anyone. Everyone is important.

First and foremost I would like to express my deepest appreciation to my supervisor Greg Hurst who is a wonderful person and one of the funniest, friendliest and the smartest people I know. He is someone you will instantly love and never forget once you meet him. Despite my poor English and lack of experience in a lab, Greg believed in me and gave me a wonderful opportunity to do PhD and work on two-spot ladybird, one of the most fascinating and beautiful organisms. Greg has been very supportive since the day I began working with him and he has guided me through all the years. I appreciate his vast knowledge and skills in many areas. I am very grateful to Greg for opening the science world for me and sharing the passion for science and nature and ladybirds! I am also grateful for his patience, for providing me with the excellent atmosphere during my PhD and for his advices and enthusiasm which made this thesis possible. Dear Greg, I really appreciate what you have done and I will be always grateful to you. Thank you Greg. You are wonderful!

I am very grateful to Amanda Minter and Sarah Trinder for their constant support and encouragement during my PhD. They are amazing friends, always ready to help. You never get bored with them. Amanda and Sarah have been very friendly, funny and thoughtful. I appreciate their kindness and understanding and I admire their ability to smile despite any situation. Thank you Amanda for sharing with me the passion for coffee and cats. Thank you Sarah for sharing with me the passion for plants. Dear Sarah and Amanda, you are wonderful!

I would like to offer my special thanks to Louise Reynolds. Louise has helped me a lot during my PhD and she has been a very good friend and an amazing person. She helped me a lot during ladybird collections in Sweden. Without her help I wouldn't be able to find so many ladybirds. I could always rely on her and ask for advice.

Louise is always very funny and makes me laugh (for instance when she reads the newspaper headlines out loud in the office). Thank you Louise for being always very friendly and kind.

Thank you to Kevin Arbuckle, Rudi Verspoor and Jamie Alison. They are amazing people. They have been wonderful friends and coffee companions. I have really enjoyed conversations with them. Thank you for your support and for making me laugh, especially during long hours at work.

I would like to thank Annabel Rice and Crystal Frost for their constant support and guidance during my PhD and helping with my ladybirds. I would like to thank them for their friendship and the warmth they extended to me during my PhD. I have learnt a lot from them and I will never forget their kindness and friendliness. Additional thanks for Steve Parratt who introduced me to molecular biology and taught me laboratory techniques. I would like to thank him for his motivation, for his support especially during the first years of my PhD, and for being always so funny.

I will be forever thankful to Zen Lewis. Zen has been very supportive and helpful in providing advice many times during my PhD. She is a wonderful person, always very friendly and thoughtful. Dear Zen, thank you for your motivation, kind words and for telling amazing stories about Japan.

I would like to express my gratitude to Kayla King for her constant motivation and support. Kayla is always enthusiastic about science and her enthusiasm is contagious. I admire her positive attitude. She asks very interesting questions which lead to interesting ideas.

Thank you for those who proofread chapters: David Atkinson, Chris Corbin, Georgia Drew, Louise Reynolds and Rudi Verspoor. I appreciate their work and time. Their comments and feedback really helped to improve and finish all chapters. Thank you. I am also grateful to Anne Lizé for her help with statistical analysis.

I would like to offer my special thanks to Beth Levick, Chris Corbin Becky Jones, and Amy Eacock for being always so friendly and supportive. Thank you for the

wonderful time we spent during organizing workshops and events for children and students where we shared our passion for science. I have learnt a lot from you. Thank you for all your advices and making me laugh. I am also grateful to Chris Corbin who helped me a lot during one of my fieldwork in Sweden and with ladybird collection. I would like to thank Cassandra Raby for her enthusiasm and passion which were inspirations for me to learn more and do more. Thank you to Łukasz Łukomski and Rudi Verspoor for all 50p and £2 pound coins he collected for me. Thank you Łukasz for giving me the possibilities to talk in Polish, for your advices and for being always so funny (Dziękuję Łukasz!). I would also like to thank Vinnie Keenan for his positive attitude and making me smile.

Additional thank you to Georgia Drew, Chloe Heys, David Wallis, Gabriel Pedra, Leni Collin, Steve Price, Sam Barlow, Hazel Nichols, Nicola Young, Becci Turner, Andy Turner, SÓnia Ferreria, Olalla M. Lorenzo-Carballa , Paulina Giraldo-Perez, Karen Evans, Kieran Pounder, Yevhen Suprunenko, Susan Withenshaw, Rowan Connell, Wei Zhang, Sarah Forrester, Chris Mitchell, Julie Truman, Ewan Harney, Ewan Minter, Angela Sims, Kate Hutchence, Rowan Doff, Laura Gordon, Laura Martin, Louise Soanes, Shelly Maiden, Ellie Harrison, Christopher Lowe and Jessica Lingley for being always very kind, supportive and friendly. You are all wonderful.

I am grateful that I have met a wonderful academic staff from our Institute and I have had opportunities to learn form the best. Especially thank you to Mike Begon, Zen Lewis, David Montagnes, Steve Paterson, Andy Fenton, Ray Whitlock, Tom Price, Stewart Plaistow, Mike Speed, David Atkinson, Ilik Saccheri, Alistar Darby, Stephen Cornell, Jenny Hodgson, Raphael Levy, Arjen Van't Hof, Pascal Campagne, David Williams, Ian Goodhead, David Thomson, Neil Hall, Soraya Shirazi-Beechey, Phill Watts, Lu-Yun Lian, Igor Barsukov and Geoff Parker.

I would like to acknowledge Louise Crompton and Carole Thomas for their kindness and support, and Meriel Jones and Linda Marsh for their guidance during my PhD.

Thank you to Brian Chan and Carl Yung for their help and for being a great lab companion during long hours in the lab. I would also like to thank Arjen Van't Hof

for his advices and support in the lab. Special thanks to Mary-Jo Hoare for supporting me during my first fieldwork in Sweden and with ladybird collections.

I would like to express my gratitude to Tom Heyes for his help with maintaining aphid populations and bean plants, and for his friendliness and constant support in the lab.

I would like to thank all staff from the Store and Wash-up who really helped me during my PhD. Their great work helped to run my experiments smoothly and without any problems. Thank you for their kindness and support.

I would also like to express my gratitude to the University of Liverpool for giving me this wonderful opportunity to work here and for funding my studentship.

I would like to offer my special thanks to my flatmates Sophie, Jake, Liz, Guy, Anne, Joseph, Layla, Ben, Toni, Phil, Andy and Joe for their support and friendliness. I would also like to acknowledge Paul Elliott and his parents.

Finally I am grateful to my parents, my sister Martyna, Yann, my niece Zoé and my family who have supported me for many, many years and they have never stopped believing in me. Thank you to my lovely parents who are wonderful and good people. They showed the beauty of nature and inspired my interests in biology. They showed me how to be a good person and gave me opportunity to follow my dreams. They have been next to me all the time. Thank you! Thank you to my lovely sister who is the best sister in the world. She is amazing person and she has inspired me. She always says ‘Never ever you give up’. Thank you for all her advices, support, understanding, motivation and for being the best friend. Thank you to my little niece Zoé for her smile and for being sunshine in my life.

Table of Contents

List of Figures

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

List of Tables

Chapter 1

Chapter 2

Chapter 3

Chapter 6

Appendix

Abbreviations:

MK – Male-killing bacteria/Male-killer

OID – Ordinary Infectious disease

PCR – Polymerase chain reaction

STI – Sexually transmitted infection

Chapter 1: Introduction

1.1 Parasite and pathogen diversity

Animals are host to a number of parasites and pathogens, which play an important role in shaping the ecology and evolution of their host. Ecologically, mortality effects are such that parasitism and pathogens can be density dependent factors that regulate host population size (Woiwod and Hanski, 1992), and they may also in extreme circumstances produce local extinction. Beyond this, parasites and pathogens may affect community dynamics, and the outcome of inter-specific competition (Morris *et al.*, 2004). Where parasites act differentially by sex, they may also alter the reproductive ecology of host the species (Jiggins *et al.,* 2000).

The mortality effects that parasites and pathogens inflict upon their hosts exert a selection pressure both for the host to avoid becoming parasitized, and also to mitigate the effects of parasitism should it occur. As such, parasites and pathogens represent potent evolutionary forces. The most obvious adaptive responses to the presence of parasites are changes in the sequence and expression of genes involved in the immune system (Koella, 2009). However, adaptations to parasitism occur much more widely than this, and may include aspects of behaviour, physiology, life history, and cell biology (Schmid-Hempel and Sadd, 2009). Indeed, it is commonly thought that parasitism has a particular role in driving co-evolutionary arms races (Brockhurst *et al.*, 2014). This importance derives from the selection they put on their hosts, and the reciprocal selection their hosts places upon them. This tendency to enter perpetual co-evolutionary cycles has led to parasites being regarded as strong 'Red Queen' forces, responsible for substantial evolutionary change.

The above represents a generic view of the influence of parasites. However parasites themselves are very diverse. A core feature of parasite diversity is the variety of methods by which they are transmitted between individuals, and how they spread through natural populations (Table 1.1). Parasites and pathogens may be transmitted either horizontally (infectiously) or vertically (from a parent to their

offspring). Infectious transmission may be through direct 'generic' contact (e.g. ebola), through direct sexual contact (for sexually transmitted infections), through propagules transmitted through the environment (e.g. through sneezing), or through a vector (e.g a tse-tse fly transmitting *Trypanosoma sp.*). A parasite or pathogen may utilize one or more host species, either with direct transmission within a community of host species, or through defined 'complex' life cycles where the parasite moves between often very different host species in a defined order (e.g. schistosomes snail-human cycles).

Table 1.1: Examples of different parasite transmission routes.

Parasite	Host	Transmission	Reference
Trichomonas vaginalis	Cattle, human	Direct	Poole and McClelland, 2013
Entamoeba histolytica	Human	Contaminated water, food or faecal-oral transmission	Loftus *et al.*, 2005
Giardia duodenalis	Human, but also cats, dogs, birds	Contaminated water, food or faecal-oral transmission	Sprong *et al.*, 2009
Trypanosoma brucei	Human	Vector-borne transmission; Transmitted by tse-tse fly *Glossina ssp*	Goodhead *et al.*, 2013
Plasmodium falciparum	Human	Vector-borne transmission; Transmitted by mosquitoes	Gardner *et al.*, 2002
Toxoplasma gondii	Cat (definitive host) Rodents, birds, human (intermediate host)	Predator-prey transmission	Tenter *et al.*, 2000
Schistosoma mansoni	Human (definitive host) Snail (intermediate host)	Predator-prey transmission	Berriman *et al.*, 2009
Taenia saginata	Human (definitive host) Cattle (intermediate host)	Predator-prey transmission	Dorny and Praet, 2007

The mechanism of transmission impacts upon a parasite's ecological and evolutionary dynamics (Thrall *et al.*, 2000). A vector borne infection, for instance, has dynamics that depend on vector number and competence. The infection is selected to maintain a vector well and also to increase the chance that an infected host is fed upon by the vector (Ewald, 1983). Parasites and pathogens transmitted through complex life cycles similarly will have dynamics, and evolutionary pressures, that depend on each host species. A vertically transmitted infection requires a healthy, reproducing host in order to attain transmission. However, because transmission is maternal, these infections may select for the production and survival of daughters over sons, thus generating a parasitic phenotype (Hurst and Frost, 2015).

1.2. Sexually transmitted infections

Sexually transmitted infections (STIs) are pathogens and parasites that require sexual contact between hosts for their maintenance within a species. This definition excludes pathogens and parasites in which some sexual transmission is observed but other means of transmission drive the pathogen epidemiology. This definition does not however debar other routes of infection from being important. For instance, HIV represents an STI in that it requires sexual contact for its spread, but is additionally transmitted from mother to child during birth, and through contact with contaminated blood.

STIs are recognised in a wide range of animal species, and have been particularly well researched in mammalian and avian hosts (Lockhart *et al.*, 1996; Knell and Webberley, 2004). Our knowledge of insect STIs, especially in natural populations, needs more attention. Lockhart *et al.* (1996) presented a list of 29 insect species suffering from STIs, and Knell and Webberley (2004) expanded this to observations of 73 different STIs in 182 insect species. This is likely an underestimate of the total number of STIs in insects.

There is an apparent contrast in the nature of STIs between vertebrates and invertebrates. Whilst the majority of STIs described in mammals are bacteria and viruses, insect STIs are often fungi or multicellular parasites such as nematodes or mites (Knell and Webberley, 2004). There may be a study bias against the discovery of microparasitic STIs in insects, as these require focussed study to detect (compared to finding a fungus, nematode or mite on a host). However, sexually transmitted mites and nematodes are not known in mammals, and thus it is likely that ectoparasitic STIs are more common in insects than mammals.

The impact of sexually transmitted infections on their host is different from other kinds of diseases. Sexually transmitted infections are selected to maintain a healthy, copulating host, as this maximizes transmission opportunities. Knell (1999) suggested that sexual selection for females to choose uninfected partners further provides a selection pressure on the STI to become cryptic. He developed a model which shows that if an individual chooses a mating partner based on the characteristics of health or parasitism, then there should be selection for reduced virulence of STIs (Knell, 1999). However, STIs are more likely than other infections to have sterility impacts (Lockhart *et al.,* 1996). Sterility impacts derive in part from their position as infections of the genital tract. However, sterility may also be a product of adaptation (Apari *et al.*, 2014). For instance, preventing a female mammal from establishing pregnancy will maintain her in the mating pool, transmitting infection. Further, extracting resources from reproduction rather than from somatic survival allows the STI not to make their host unhealthy and unattractive for mating. The concept that STIs may create sterility as an adaptive phenotype is supported by the observation that ectoparasitic STIs in ladybirds lead to sterility of their female host in spite of having no direct interaction with the reproductive system (Hurst *et al.*, 1995).

The dynamics of sexually transmitted infections are also commonly considered to be distinct from those of 'Ordinary infectious diseases' (OIDs). Because STIs spread as a result of sexual contact, the density of the host is considered to be rather less important in determining transmission opportunities. Instead, transmission opportunities are determined by the frequency of mating, a factor commonly

thought to be relatively independent of the number of contacts a host has with other conspecific individuals. A consequence of this is that, unlike an OID, there is either no, or a very low, threshold host density required for an STI to establish in a population. Furthermore, there is no density dependent regulation of the host population by the infection, and theory suggests sterilizing STIs are thus more likely to make their host go extinct (Lockhart *et al.*, 1996; Thrall *et al.*, 1997; Thrall *et al.*, 2000).

Empirical verification of these principles is, however, rare. Indeed, the spread of STIs to high prevalence and host extinction is avoided where promiscuous individuals mate with other promiscuous individuals. When this occurs, the STI circulates predominantly in just one part of the population, as seen for gonorrhoea in humans (Hethcote and Yorke, 1986). It is also likely that STIs do show some density dependence. When hosts are very rare, mating rates may be lowered. Laboratory simulation of STI transmission in *Adalia bipunctata* indicates that STI transmission rate is modulated, at least in part, by host density (Ryder *et al.*, 2005).

STIs present an interesting case where the dynamics of the disease is affected by mating system. Different intensities of sexual selection in male and females, for instance, are predicted to be reflected in sex-biased patterns of infection (Ashby and Gupta, 2013). Where reproduction is focussed on a few males, these males represent super-spreaders which then pass infection into females. The less successful beta males, in contrast, are predicted to have low prevalence of infection. This scenario is observed in the sub-tropical eucalypt beetle, *Chrysophtharta cloelia*. This species shows sexual selection for large male size (Nahrung and Clarke, 2007). The sexually transmitted *Parobia captivus* mite, in accordance with prediction, is found more commonly on large males than small, and also more commonly on female hosts compared to male (Nahrung and Allen, 2004; Nahrung and Clarke, 2007).

It has been widely conjectured that mating system will evolve in response to the presence of an STI. Thrall *et al.*, (1997) and Kokko *et al.* (2002) postulate that presence of an STI will select for a reduced mating rate. The prediction is that female individuals, being more impacted by sterility and with less fitness gain from mating multiply, will be strongly selected to reduce mating rates as a means of reducing STI exposure. Boots and Knell (2002), considering the same issue, predicted selection would produce a mix of individuals showing risky and safe sexual behaviour, rather than monogamy. They argue 'risky behaviour' is maintained as its cost reduces when risky individuals are rare. As monogamy becomes more common, they argue that the STI becomes rare, enabling the risky behaviour to persist.

It has also been suggested that STIs may select for mate choice, and for tolerance/avoidance behaviours. Attempts to uncover mate choice in favour of uninfected individuals have largely failed (Knell, 1999; Webberley *et al.*, 2002), probably because STIs are strongly selected to be cryptic. Elsewhere, post-copulatory behaviours, such as grooming and masturbation, have been suggested as a means of preventing infection from becoming established (Waterman, 2010). Finally, Nunn *et al.* (2002) argue that the association between promiscuity and immune system activity amongst primate species is driven by the increased prevalence of STIs in promiscuous species.

1.3 Ladybird model system for STI study

The two-spot ladybird *Adalia bipunctata* is a generalist aphid predator found throughout the Holarctic region. It has historically been common in Europe, especially in Western and Central Europe, and also in Central Asia and North America (Majerus, 1994; Omkar and Pervez, 2005). Numbers have however recently declined following the introduction of the 'invasive' *Harmonia axyridis* from Asia (Koch, 2003; Brown *et al.*, 2008). Two-spot ladybirds live in many habitats, but the preferred environments are urban and suburban areas. In these places, they can be found on lime trees (*Tilia sp.*), sometimes willows (*Salix sp.*) or birches (*Betula sp.*), and also on wild rose bushes (*Rosa sp.*) and nettles (*Urtica dioica*) (Majerus, 1994).

The two-spot ladybird has been established as a useful species for ecological and evolutionary studies. Initially, the highly polymorphic elytral forms (Figure 1.1) led to it being used as a model for understanding the evolutionary forces underlying phenotypic variation in natural populations. It is considered aposematic, though less toxic than other sympatric ladybirds such as *C. septempunctata* (Marples *et al.*, 1989; Marples *et al.*, 1994). Patterns of mate choice (Majerus *et al.*, 1982) and thermal melanism (Brakefield and Willmer, 1985) were considered as drivers in the spatial and temporal variation in colour pattern observed in natural populations.

More recently, the species has been used as a 'model organism' for three different areas of enquiry: promiscuity/sexual selection, heritable symbiont impacts, and interactions with natural enemies, particularly sexually transmitted infections. A core hypothesis within this thesis is that these three areas of research may be related: promiscuity/sexual selection driving STI epidemiology, STI epidemiology driving mating system biology, and heritable microbes potentially modifying both. I thus briefly review each field of research, before combining these studies to better understand these interactions.

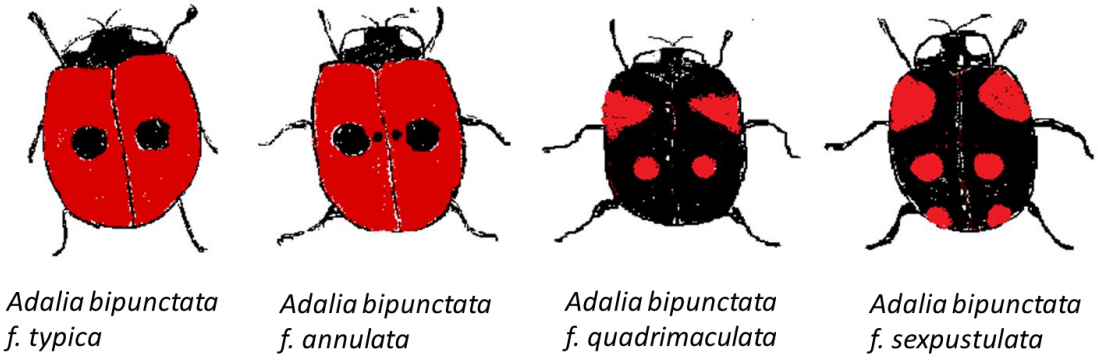


Figure 1.1: The most common forms of two-spot ladybird *Adalia bipunctata.*

1.3.1 Interactions with heritable symbionts

Heritable microbes are viruses, bacteria, fungi and protists that pass to the next generation during reproduction. The microbes are most commonly maternally inherited, passing from a female into her progeny, but not from a male host. Heritable microbes are now known to be common in arthropods, with over half of species carrying one or more infections. Their interactions with the host are diverse (For examples, see Table 1.2).

Table 1.2: Examples of interactions between host and heritable microbes.

Heritable symbiont	Host	Impact on host	References
Spiroplasma ixodetis	*Adalia bipunctata*	Killing males	Hurst *et al.*, 1999a
Rickettsia	*Adalia bipunctata*	Killing males	Hurst *et al.*, 1999a
Wolbachia pipientis	*Adalia bipunctata*	Killing males	Hurst *et al.*, 1999b
Spiroplasma	*Drosophila neotestacea*	Symbiont-mediated protection against nematode infection; Mutualism with *Wolbachia*	Jaenike *et al.*, 2010b
Wolbachia	*Drosophila neotestacea*	Mutualism with *Spiroplasma*	Jaenike *et al.*, 2010a
Wolbachia	aphids	Symbiont-mediated protection against parasitic wasp	Oliver *et al.*, 2003
Buchnera aphidicola	aphids	Provides essential nutrients for aphids	Douglas, 2010
Spiroplasma	*Drosophila melanogaster*	Killing males	Montenegro *et al.*, 2005
Wolbachia pipientis	*Drosophila melanogaster*	Symbiont-mediated protection against RNA virus infection	Hedges *et al.*, 2008
Wolbachia pipientis	*Hypolimnas bolina*	Killing males	Horrnet *et al.*, 2006
Arsenophonus nasoniae	*Nasonia vitripennis*	Killing males	Huger *et al.*, 1985
Wolbachia	*Nasonia vitripennis*	Cytoplasmic incompatibility	Werren *et al.*, 1995
Cardinium	Planthopper	Feminization	Nakamura *et al.*, 2009

Adalia bipunctata is one of the first recorded cases of maternally inherited male-killing elements. Lineages of ladybirds producing daughters only were recorded by Lus in 1947 in Russia. Sex ratio bias was noted to be associated with low egg hatch rate, making male death the likely cause of the sex ratio skew. Inheritance was strictly matrilineal (Lus, 1947). The trait was further explored nearly fifty years later in the UK populations (Hurst *et al.,* 1992; Hurst *et al.,* 1993a). Here, 7-20% of female individuals carried the male-killing trait, which was revealed to be sensitive to antibiotics, implying a bacterial origin. Male-killing was also found to be associated with the presence of a *Rickettsia* bacterium (Werren *et al.,* 1994).

This maternally inherited symbiont is transmitted from females (mothers) to their daughters and sons but only daughters survive and hatch from eggs. Sons are killed during embryogenesis, they are an evolutionary dead end for cytoplasmic genes (Hurst, 1991; Hurst and Majerus, 1993b; Hurst and Jiggins, 2000). The commonness with which infection is observed in ladybirds is thought to be associated with the presence of sibling egg cannibalism in coccinellids, which means that dead male eggs are consumed by their hatching sisters, which is advantageous for a maternally inherited agent. The presence of the male-killer increases the survival prospects of host female larvae in two ways: firstly by increasing the food resources available to hatching female larvae that carry the infection through consumption of the dead male eggs, and secondly by decreasing the competition between sisters for food (Hurst *et al.,* 1992; Hurst and Majerus, 1993b).

Interestingly, later studies and sequencing analysis have revealed that male-killing bacterium found in the UK populations of *Adalia bipunctata* was different from male-killing bacterium found in the Russian populations. The Central European populations were observed to carry a male-killing *Spiroplasma* alongside another male-killer, *Rickettsia* (Hurst *et al.*, 1999a), and samples from Russia revealed the presence of male-killing *Wolbachia* (Hurst *et al.,* 1999b). The Moscow population indeed carries all three male-killing symbionts (Majerus *et al.,* 2000). Further surveys in Sweden indicated that the Stockholm population carried a high prevalence of *Spiroplasma*, with around 40% of females carrying this symbiont (Zakharov and Shaikevich, 2001). More complete sampling indicated variation in the

prevalence of male-killing bacteria in Sweden, with *Spiroplasma* common and dominant south of 61˚N, but lower prevalence of this bacterium, alongside *Rickettsia*, being found north of this area (Tinsley, 2003: see Figure 1.2).

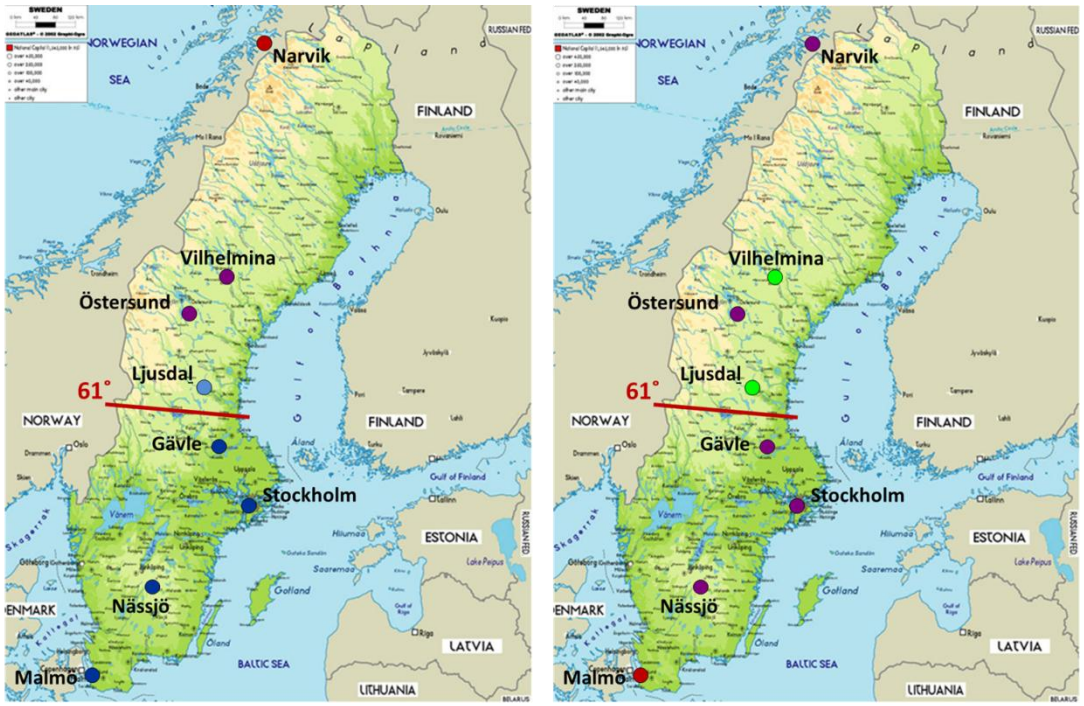


Figure 1.2: The occurence of *Spiroplasma* male-killing bacteria in Scandinavian countries between 2000-2002 (Tinsley, 2003). Sampling regime by colour: ● *Spiroplasma* prevalence was higher than 30%; ● *Spiroplasma* prevalence between 10-30%; ● prevalence of *Spiroplasma* reached up to 10% of females in *Adalia*; ● *Rickettsia* prevalence higher than 30%; ● *Rickettsia* prevalence between 10-30%; ● *Rickettsia* prevalence up to 10% of female *Adalia* populations; ● Absence of both *Spiroplasma* and *Rickettsia* (Map source: www.in-sweden.co.uk/physical-map-of-sweden.html).

To date, we know little about the forces driving variation in heritable microbe presence in natural populations. Spatial variation in male-killer incidence/prevalence is common (Majerus *et al.*1998 *Harmonia axyridis*; Chang *et al.,* 1991 *Gastrolina depressa*; Charlat *et al.,* 2005 *Hypolimnas bolina*), but generally unexplained. This deficit applies to *A. bipunctata* in Sweden, where spatial variation

exists but is not explained. What is known is that *Spiroplasma* infection has an important effect on the ladybird population. It creates a distortion in the ladybird population sex ratio, which can become very female-biased (Hurst *et al.,* 1993a; Ryder *et al.,* 2014). This population sex ratio bias is likely to alter *A. bipunctata*'s mating system, and in so doing affect the dynamics of any sexually transmitted infection.

1.3.2 Promiscuity and sexual selection

Two-spot ladybirds are promiscuous. Females and males mate many times with different partners throughout their lives in both the wild and in the laboratory (Majerus, 1994; Haddrill *et al.,* 2008). When a male meets a female, he climbs on her back, without apparent courtship. She may then accept or reject him, with rejection made more likely when the female is poorly fed (Majerus, 1994; Perry and Rowe, 2008b; Perry *et al.*, 2009). If a female rejects the partner, she usually runs away or lifts her abdomen and pushes him away or she turns on her back or she drops from the top of the lid.

When mating happens, it takes approximately 140±65 min (Perry and Rowe, 2008a). During copulation males transfer sperm with seminal fluids via one or more spermatophores. Just after copulation (usually 2-4 minutes later) most of the females (90% of matings) release this spermatophore and consume it (>90% of females consume more than half of the spermatophore and around two-third of them consume the entire spermatophore) (Perry and Rowe, 2008a,b; Perry, 2011; Perry and Tse, 2013). The consumption of the spermatophore promotes egg production, oviposition and increases female resistance to remating with another partner (Perry and Rowe, 2008b). Thus, spermatophore consumption is advantageous to the male, as it increases the number of progeny he sires with a particular female.

1.3.3 Sexually transmitted infections

The promiscuity of *A. bipunctata* is associated with the presence of sexually transmitted infections. Two infections are considered to be transmitted during host copulation. First, there is the Laboulbeniales fungus *Hersperomyces virescens*. This ectoparasitic fungus lives within the ladybird cuticle, from where fruiting bodies appear. An intense infection is associated with the ladybird host appearing with a 'furry' yellow coat. Sexual transmission has been inferred as being important from the position of the infection on the ventral side of the male abdomen and the dorsum of females, reflecting sexual contact points. However, other routes of infection have not been excluded. Prevalence of the infection can be locally high (e.g. in London in spring when even > 50% of adult ladybirds carrying the fungus infection: Welch *et al.* 2001) and there is some association with urban environments (Webberley *et al.*, 2006b).

Second, there is *Coccipolipus hippodamiae* mites, the subject of this thesis. The interaction between two-spot ladybirds *Adalia bipunctata* and the parasitic mite *Coccipolipus hippodamiae* is one of the best studied models for STI biology (Hurst *et al.*, 1995; Webberley *et al.*, 2002; Webberley *et al.*, 2004; Knell and Webberley, 2004; Webberley *et al.*, 2006a; Webberley *et al.*, 2006b; Ryder *et al.*, 2013; Ryder *et al.*, 2014). *Coccipolipus hippodamiae* (family *Podapolipidae*) is principally an ectoparasite of *A. bipunctata*, with infection also observed on three other European ladybird species: the ten-spot ladybird *Adalia decempunctata*, *Oenopia conglobata* and cream-spot ladybird *Calvia quatuordecimguttata.* However, the prevalence of mite infection on *A. decempunctata*, *Oenopia* and *Calvia* species is low (Webberley *et al*., 2004). Mites were described on the two-spot ladybirds for the first time in the USA by Husband (Husband, 1981) and then in Europe (in the Russian populations) by Hurst (Hurst *et al.,* 1995).

The interaction between the mite and two-spot ladybird is typical of insect-STI systems. The mite infects ladybird adults and is transmitted between partners during copulation. There is no apparent 'mate choice' to avoid mating partners based on infection status (Webberley *et al.*, 2002), and laboratory assays indicate

that mite transmission occurs readily. Where there are larval mites on just one of the two mating individuals, mite infection is transferred to the other party on around 90% of occasions.

The mite itself lives underneath the ladybird elytra, where it feeds on ladybird haemolymph. The adult mite is sessile and lays eggs (around 2 per day) which hatch into larvae after 3-4 days. Only female larvae can move between hosts during copulation (Hurst *et al.,* 1995; Webberley *et al.,* 2004; Webberley *et al.,* 2006a). The mite is predominantly sexually-transmitted however laboratory observations have recorded that larval mite transmission, between aggregated ladybirds, during overwintering is also possible but rather rare (Webberley and Hurst, 2002). Furthermore a mite cannot survive for long without its host, therefore when a mite leaves its host it has to find a new host very quickly.

The mite is harmful for *Adalia bipunctata*. It develops quickly under the elytra of both sexes (Webberley *et al.,* 2002; Webberley *et al.,* 2004; Webberley *et al.,* 2006a). Mite infection sterilizes female ladybirds after about 10-14 days of infection and those host females continue to lay eggs which characteristically shrivel and do not hatch (Hurst *et al.,* 1995). There is also some mortality effect of mite infection, with infection with this parasite reducing overwinter survivorship, especially for male beetles (Webberley and Hurst, 2002).

One of the most pronounced features of the mite-*Adalia* system is the presence of recurrent seasonal epidemics associated with mating activity. In populations where the mite is present, around 10% of ladybirds emerge from overwintering carrying mite infection. Ladybirds are highly promiscuous and start their mating activity in April-June (depending on latitude). They move between host plants and this allows ladybirds to mix (Brakefield, 1984). The movement and high mating activity speed up spread of the mite epidemic in spring, with over 90% of ladybird being infected after around 10 weeks (Webberley *et al.*, 2006a). In populations where a male-killing symbiont is present (e.g. *Spiroplasma* in Stockholm, Sweden), the sex ratio is female-biased with a 3:1 or 4:1 sex ratio. In this population males become infected

with mites earlier than females, in contrast to places where a male-killing bacterium is absent (for instance Toruń, Poland) (Ryder *et al.*, 2014).

The mite is thus a formidable ecological force: it infects nearly all adult individuals where it is present, and sterilizes its female host. However, there is little evidence of resistance to the mite in natural populations. Nevertheless, mite presence shows strong geographic patterning, being present in central and southern Europe, and absent from western and very northern regions (Webberley *et al.*, 2006b). Some of the most profound presence/absence variation is found in Sweden. Surveys by Tinsley (2003) recorded the mite being present in the south of Sweden (south of 61˚N) in Malmö, Stockholm and Gävle but being absent in northern Sweden (Ljusdal, Östersund and Vilhelmina) and also in Nässjö, the only southern location recorded free from mites (Figure 1.3). Phenological change in the ladybird has been hypothesized as the major driver of presence/absence of the mite (Hurst *et al.* 1995), although data are, to date, lacking.

Figure 1.3: The occurence of the mite *C. hippodamiae* in Scandinavian ladybird populations between 2000-2002 (Tinsley, 2003). Orange ● places where the mite is absent. Green ● places where mite is present (Map source: www.in-sweden.co.uk/physical-map-of-sweden.html).

1.4 Aims of thesis

Research into symbionts, STIs and mating system in *A. bipunctata* has largely been conducted independently. However, there are obvious potential interactions between the three areas of study (Figure 1.4). Most obviously, mating system biology affects the spread of a sexually transmitted infection, as the pattern of sexual contact determines transmission opportunities (Ashby & Gupta, 2013). Reciprocally, theory states that presence of an STI may select for an alteration in mating system, promoting a reduction in promiscuity (Kokko *et al.,* 2002). Beyond this interplay, male-killing symbionts affect mating system biology through altering population sex ratio, and thus the rate at which males and females make contact (Ryder *et al.*, 2014). This then may impact upon STI dynamics. A further potential

interaction is through protection – symbionts are known to alter susceptibility to natural enemies in a number of systems (Haine, 2005). Any change in individual susceptibility to the mite associated with symbiont presence would alter the dynamics of both mite and symbiont.

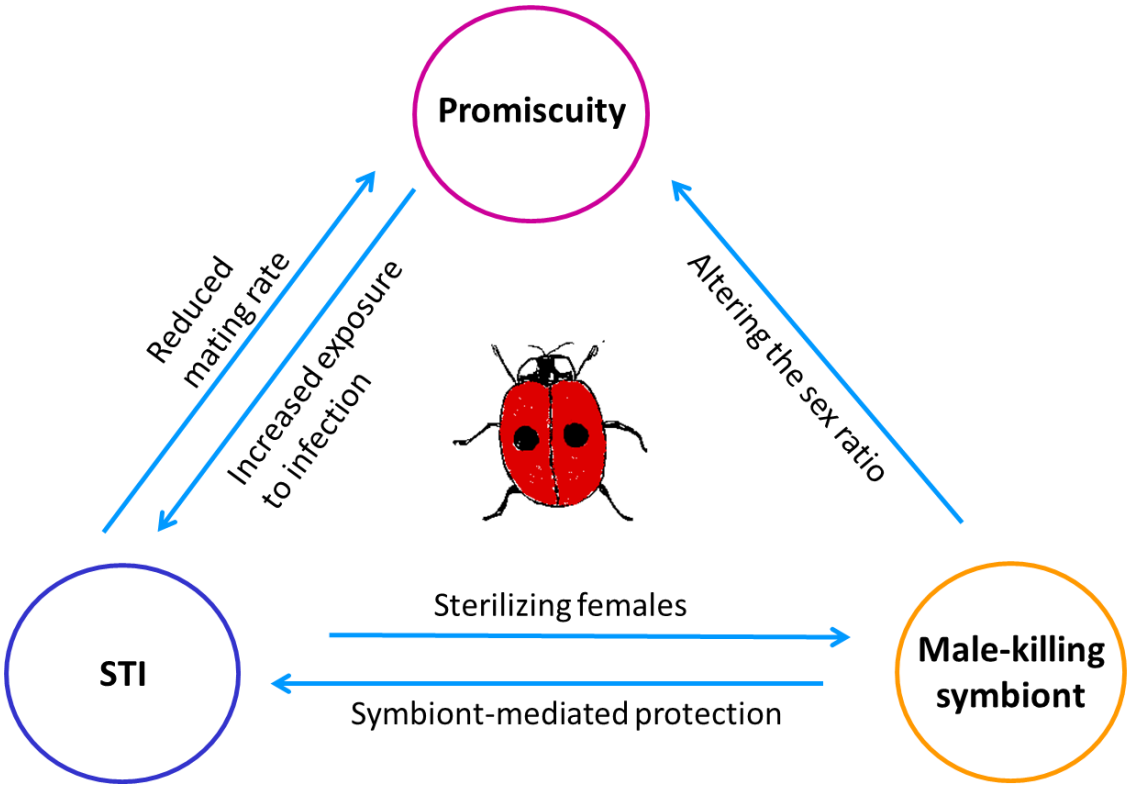


Figure 1.4: Potential interactions between male-killing symbionts, promiscuity and STI. Each arrow represents a direct potential impact of one factor on the dynamics of another. These impacts may be either ecological (e.g. sex ratio alters promiscuity) or evolutionary (presence of an STI may select for changes in promiscuity).

There is thus a case for examining the biology of the STI in the wider context of symbiont presence and mating system variation. In order to conduct such an investigation, it is helpful to have variation in the presence/absence of the mite, and the symbiont. The Swedish population of *A. bipunctata* presents a good opportunity for this. Past studies have indicated that *Spiroplasma* bacteria and *Coccipolipus*

hippodamiae are quite common in the populations in the south of Sweden, but are absent (the mites) or low in prevalence (*Spiroplasma*) in the north. The first comprehensive survey of these parasites was conducted by Tinsley (2003), who observed about 40% prevalence of the *Spiroplasma* in populations south of latitude 61°N, along with presence of the mite below this latitude in all populations (expect of one from Nässjö). In contrast, the mite was always absent and *Spiroplasma* prevalence was very low north of this line of latitude.

This thesis focuses on the incidence and epidemiology of the mite, and its interplay with host mating system and symbiont presence. The aims are:

a) To understand how mite presence/absence is determined in Swedish populations of *A. bipunctata*.

b) To determine if mite presence is associated with changes in mating system, in particular a reduction in host promiscuity.

c) To establish the likely influence of population sex ratio bias associated with male-killing bacteria on the dynamics of the STI.

d) To determine whether there is a direct link between *Spiroplasma* infection and host susceptibility to mite infection, and if there are other potential links mediated through longevity.

1.5 Thesis outline

In Chapter 2, I examine the stability of mite and *Spiroplasma* clines over time. The rationale for this study is that constant biological/ecological forces are likely to produce stability in incidence and prevalence patterns over time, and that ecological variation (for instance range shifts associated with climate change) or evolutionary change (such as host-parasite coevolution) would alter this pattern. In this chapter, I thus repeat previous surveys to answer a single core question: Is the

pattern of mite and *Spiroplasma* presences stable (indicating an unchanging biological/ecological basis to the pattern), or have it changed over 10 years?

In Chapter 3, I then examine the biological and ecological factors that determine the incidence of the mite in Swedish *A. bipunctata*, with particular focus on the determinants of mite absence. One of the challenges arising from environmental change is to predict how the population size and range of insect pests and their natural enemies will respond to climate change. We can approach issues like this in a principled fashion through understanding the environmental factors that limit the current incidence and prevalence of pests and the natural enemies that control pest numbers. Previous studies have shown that the mite is absent in the northern Swedish ladybird population but it has been common in the south (except one place Nässjö where mites were not observed). Two hypotheses are tested: a) Is the cause of mite absence an inability of ladybirds from the north of Sweden to become infected with mite/transmit it to onward? b) Does the phenology of northern ladybirds prevent mite transmission between generations, such that the mite cannot persist in the population?

In Chapter 4, I extend previous studies examining the impact of the mite on the evolution of host mating system. It has been predicted, on the basis of mathematical models, that STIs should select for an alteration in mating system, with females in particular being selected to have reduced mating rate (Kokko *et al.*, 2002). I present the first test of this hypothesis, by examining whether rejection behaviour (that prevents mating) occurs more commonly when the ladybirds derive from a population where the STI is present, compared to one where it is absent.

In Chapter 5, I examine whether heritable symbionts may modulate mite dynamics through altering the biology of their host. Recent work (see above) has highlighted the role of *Spiroplasma* in *Drosophila* and aphids as a defensive symbiont, making the host resistant to attack by parasitic wasps or nematodes. I ask if this symbiont has any impact on host ability to acquire or transmit mite infection onward, and whether there is any amelioration of mite phenotype (sterility). Further to this, I ask whether *Spiroplasma* influences longevity in field collected beetles, as recently

noted in laboratory study (Elnagdy *et al.*, 2013). An effect such as this would be important for mite dynamics, as it would represent a phenological alteration (Chapter 3) that may affect mite dynamics.

In Chapter 6, I examine the potential for indirect influence of the *Spiroplasma* on mite dynamics, mediated through skewed population sex ratio. My thesis is that where the male-killer is common, population sex ratios are female-biased. Individual male ladybirds then have a higher mating rate than female ladybirds. This produces higher exposure to the STI, reflected in early epidemics of the STI on male than female host ladybirds. I will test two hypotheses: a) Sex ratio bias may reduce male to female transmission of the mite. When males mate commonly, the supply of infective larval mites may limit transmission. This effect would increase sex-biased prevalence. b) Sex ratio bias may increase female mating rate if males that mate multiply transfer less ejaculate, and make females less refractory to remating. This effect would increase the epidemic speed in both males and females.

My thesis ends with a discussion of the results, in which it is concluded that sexually transmitted infections will be less common generally on species that live near the poles and that the dynamics of these infections may commonly be altered by symbionts. It is also noted that a remaining major gap in knowledge is that we simply do not understand what drives the pattern of symbiont incidence in this, and indeed many other, species.

Chapter 2: Stability of *Spiroplasma* and mite clines over time

1. The two-spot ladybird *Adalia bipunctata* is a host to heritable male-killing bacteria and a sexually transmitted mite *Coccipolipus hippodamiae*, both of which impact on host reproduction.

2. Previous studies in Sweden noted geographical variation in the incidence of these parasites in the period 2000-2002, with mite and male-killing *Spiroplasma* being absent north of 61˚N.

3. Stability of this pattern would indicate a persistent underlying ecological driver to mite and *Spiroplasma* presence. In this chapter I therefore investigated if this discontinuity in incidence persists 10 years after the initial survey. In addition to the presence of *Spiroplasma* and mite, presence of other male-killers *Rickettsia* and *Wolbachia* were also examined.

4. The pattern of mite presence/absence remained identical in 2011 to that observed in 2000-2002. The overarching pattern of male-killing *Spiroplasma* distribution remained, with presence at high prevalence in southern populations and low prevalence in northern populations. However, there was a northward shift in the boundary between high and low prevalence populations.

5. Investigation of other male-killing symbiont presence revealed little change in *Rickettsia* distribution, which remained presence at low prevalence throughout the country. In contrast, there was evidence for *A. bipunctata* carrying *Wolbachia* infections, which had not been recorded in the previous study.

6. We conclude that the stable distribution of the mite indicates a persistent underlying ecological factor that determines its incidence. The pattern of male-killing *Spiroplasma* indicates a shifting boundary, with an underlying ecological factor that has altered through the period of study.

7. Future work should establish the nature of the ecological determinants of these patterns. It is conjectured a) changes in ladybird phenology with latitude determine *C. hippodamiae* presence/absence b) compressed breeding seasons and high food availability in northern climes may be associated with reduced prevalence of male-killing *Spiroplasma*.

2.1 Introduction

The two-spot ladybird (*Adalia bipunctata*) is host to a variety of parasites that affect host reproduction. As outlined previously, the haematophagous ectoparasitic mite *C. hippodamiae* utilizes copulation as a transmission opportunity, and makes female hosts infertile. Vertically transmitted bacterial symbionts are also common. *Adalia bipunctata* carries four different strains of male-killing bacteria: *Spiroplasma* (Hurst *et al.,* 1999a), *Rickettsia* (Werren *et al.,* 1994) and two strains of *Wolbachia* (Hurst *et al.,* 1999b). These parasites represent a strong ecological force. Where present, the STI reaches very high prevalence, thus impacting on host reproduction (Hurst *et al.*, 1997). Past records suggest male-killing bacteria may also be sufficiently common to produce population sex ratio biases, which will thus impact on mating system biology (Ryder *et al.*, 2014).

Broad surveys of these parasites have suggested their incidence varies over space. The mite is absent from western and northern populations in Europe (Webberley *et al.,* 2006b). Areas of France and Holland that are close to the Atlantic are uninfected with the mite, as are UK populations. Infections with male-killing bacteria are found throughout Europe, but the strain of male-killer observed varies spatially. In the UK and Denmark and in Kyrgyzstan in Asia only *Rickettsia* has been recorded (Hurst *et al.,* 1992; Zakharov *et al.,* 1996; Hurst *et al.*, 1999a). In Germany, Poland and Sweden, *Spiroplasma* and *Rickettsia* have been found (Hurst *et al.,* 1999a), and more easterly in Moscow and St. Petersburg, *Spiroplasma*, *Rickettsia* and *Wolbachia* (Zakharov *et al.,* 1996; Majerus *et al.,* 2000).

Past work has identified presence/absence boundaries for both the mite and male-killing bacteria in Sweden. Past study by Tinsley (2003) has suggested that the prevalence of male-killing bacteria and mites changes from the north to the south. Tinsley surveyed populations from the south of Sweden (Malmö 55˚35'N, 13˚02'E) on a N-S transect through to Narvik (68˚25'N, 17˚33'E) on the Norway/Sweden border in the Arctic Circle (Figure 2.1; material collected is detailed in the appendix Tables A2.3, A2.4 and A2.5). Surveys in 2000, 2001 and 2002 indicated *Spiroplasma* was found at high prevalence (51.8% in Gävle, 38.3% in Stockholm and 35.5% in

Nässjö) in the south of Sweden (south of latitude 61°N), but was rare north of this latitude. *Rickettsia*, in contrast, was at low prevalence (from 1.22% in Narvik to 25.3% in Ljusdal) throughout. The mite was absent north of 61˚N, and present in more southerly regions, with the exception of Nässjö, a city in the interior of Sweden which lies 375 m (1230 ft) above sea level. The dissimilarity between south and north in both distributions was not gradual, but rather a rapid transition. This suggests that there is a hard barrier to the spread of these infections in certain places.

In this chapter, I aim to assess whether the stability of the patterns is observed in Sweden. Stability of incidence/prevalence would indicate a consistent driver of incidence/prevalence pattern, and would contrast with dynamic variation, which would indicate either changing ecological circumstances or co-evolutionary interactions.

My approach is to complete the same transect as completed by Tinsley (excepting Narvik), and compare the prevalence and incidence of mite and male-killers 10-12 years after the original survey.

Figure 2.1: Collection sites of *Adalia bipunctata* across Scandinavia visited by Tinsley between 2000 and 2002. Sampling regime by colour: ● samples in Autumn 2000; ● samples from Spring 2000 to Autumn 2002; ● samples in Spring 2001; (Tinsley, 2003; Map source: www.in-sweden.co.uk/physical-map-of-sweden.html).

2.2 Material and methods

2.2.1 Collection of material

Two-spot ladybirds (*Adalia bipunctata*) were collected across three years (2011, 2012 and 2013) from Swedish urban habitats (city centres and suburbs). The locations sampled paralleled those visited by Tinsley, with the exception of Narvik (the most northerly site) which was not visited (Figure 2.2). Samples collected are detailed in Table 2.1 (2011), 2.2 (2012) and 2.3 (2013). To this systematic collection was added samples of ladybirds from Helsinki, Finland (July/August 2011 collected by Dr Emily Hornett) and Tartu, Estonia (April 2012 collected by Prof. Toomas Tammaru).

South of 63˚N, *Adalia bipunctata* were mostly found on lime trees (*Tilia sp.*, especially small-leaved lime tree *Tilia cordata*). North of this latitude (Östersund, Vilhelmina), lime trees were not present, and birches *Betula* represented the most common habitat. Where possible ladybirds were sampled from the same tree and bush species within a habitat to minimize ecological variance between collected populations (it is known that habitat type influences STI prevalence: Ryder *et al.,* 2013).

Figure 2.2: Collection sites of *Adalia bipunctata* in Sweden, Finland and Estonia between 2011 and 2013. Sampling regime by colour: ● samples in 2011 (weekly between May and July), twice in 2012 and 2013 (May and August); ● samples in 2011 (June/July), 2012 (August) and 2013 (August); ● samples twice in 2011 (June and July) and once in 2012 (August); ● once in 2011 (June/July); ● additional samples from collaborators: Helsinki (2011: only females) and Tartu (2012: females and males) (Map source: www.in-sweden.co.uk/physical-map-of-sweden.html).

Ladybirds were collected from trees by beating and from bushes by hand collection. Collected individuals were transferred to 1.5 ml Eppendorf tubes (Figure 2.3). Ladybirds that were caught as single individuals were confined singly, to prevent any mite contagion during storage. If a mating pair landed on the beating tray, ladybirds were not separated but transferred together into the Eppendorf.

Figure 2.3: Two-spot ladybirds in Eppendorf tubes (source: the Swedish website: http://www.ltz.se/jamtland/ostersund/nyckelpigsforskare-letar-orsaken-till-barnloshet).

Table 2.1: *Adalia bipunctata* samples collected in Sweden in 2011 with additional data from Helsinki (Finland) in 2011. Sites are listed in order of declining latitude, with northerly samples at the top and southerly samples at the bottom of the table.

Site	Date of collection	Females	Males	Host plants
Vilhelmina	3/7	19	9	*Betula sp.*
Östersund	4/7	22	29	*Betula sp.*
Ljusdal	17/6 28/7	56 62	33 38	*Tilia sp.,* *Rosa sp.*
Gävle	4/6 8/7	147 62	53 38	*Tilia sp.*
Helsinki (Finland)	July/August	40	N/A	N/A
Stockholm	20/5-28/7	2232	847	*Tilia sp.*
Nässjö	12/6 9/7	73 105	35 95	*Tilia sp.,* *Rosa sp.*
Malmö	11/6	126	74	*Tilia sp.*

Table 2.2: Details of *Adalia bipunctata* samples collected in Sweden in 2012 with additional data from Tartu (Estonia) in 2012. Places are listed in order of declining latitude, with northerly samples on the top and southerly samples at the end of the table.

Site	Date of collection	Females	Males	Host plants
Östersund	8/8	52	47	*Betula sp.*
Ljusdal	9/8	56	36	*Tilia sp.,* *Rosa sp.*
Gävle	9/8	42	12	*Tilia sp.*
Stockholm	22-24/5 11-12/8	334 172	183 67	*Tilia sp.*
Tartu (Estonia)	April	24	7	N/A
Nässjö	10/8	60	16	*Rosa sp.*

Table 2.3: Details of *Adalia bipunctata* samples collected in Sweden in 2013. Populations are listed in order of declining latitude, with northerly samples on the top and southerly samples at the end of the table.

Site	Date of collection	Females	Males	Host plant
Östersund	14/8	6	3	*Betula sp.*
Stockholm	21-24/5 12-13/8 +15/8	123 244	51 149	*Tilia sp.*

2.2.2 Assessing the mite *Coccipolipus hippodamiae infection status on ladybirds*

The sex of ladybirds was ascertained as described by Randall *et al.* (1992), with males identified by the presence of a notch in the final abdominal segment, and broad flexure bands between abdominal segments. Ladybirds were scored for mite presence within 24 hours of collection following Hurst *et al.* (1995). To this end, each ladybird was placed with care on its pronotum on Blu-tac™ and examined under a binocular microscope. Each elytron was carefully unfolded using a pin to expose the underside, and examined under x40 magnification. The presence/absence of mite infection was noted, and where infection was present, the intensity of mite infection estimated (number of mite adults, larvae, and eggs). An individual was deemed to be infected if any mite stage was present, and infectious if mite adults, eggs and larvae were all present. Records of mite presence/absence were compared to data from 2000-2002. Because the mite undergoes annual epidemics from May-July, followed by dilution as new generation adults emerge, comparison of prevalence between samples was not appropriate, as these are expected to differ between samples collected at different phases in the epidemic.

2.2.3 Establishing presence/absence of heritable microbes

Female ladybirds collected above were transferred to separate 1.5 ml Eppendorf tubes and preserved in 100% ethanol. DNA was extracted and the presence of male-killing bacteria then determined using diagnostic PCR assays.

2.2.3.1 DNA Extraction using the Promega Wizard® Genomic DNA Purification Kit

DNA was extracted from ladybirds using Promega Wizard Protocol designed for isolation DNA from animal tissue. To this end, whole individual ladybird females were added to 1.5 ml Eppendorf with 300 µl of room temperature Nuclei Lysis Solution and homogenized with sterile pestles until the ladybird cells were broken. The material was incubated at 65˚C for 30 minutes before addition of 100 µl of Protein Precipitation Solution. The tubes were then vortexed and chilled on ice for 5

minutes before centrifugation at 14,000 *x g** for 4 minutes. Supernatant was transferred to a new tube and DNA precipitated by adding 300 µl of room temperature isopropanol (Propan-2-ol). Following gentle mixing by inversion, samples were centrifuged at 14,000 *x g** for two minutes. Supernatant was removed and the pellet washed by addition of 300 µl of room temperature 70% ethanol and centrifugation at 14,000 *x g** for two minutes. After this stage, ethanol was carefully removed and the pellet containing DNA left to air-dry the pellet for 15 -60 minutes. The DNA was then resuspended in 60 µl of sterile distilled (MilliQ) water and incubated overnight in the fridge at 0.4˚C. Prepared DNA samples were either used directly in assays, or if not used within 24 hours, stored at -20˚C prior to assay.

2.2.3.2 Screening ladybird DNA for presence of male-killing bacteria by molecular reactions

A three-step approach to assessing male-killing bacteria presence was adopted. First, the quality of extracted DNA template was tested using a PCR assay based on CO1 mtDNA, present in all insects. Samples passing this QC were then subject to separate PCR assays for the presence of *Spiroplasma*, *Rickettsia* and *Wolbachia*, using previous methodologies. Finally, amplicons from a sample of positive individuals for each infection were sequenced to ensure the current material reflected historical records of symbiont identity.

The quality of extracted DNA was initially tested using general insect primers LCO/HCO that amplify a 610-bp fragment of the insect cytochrome oxidase mitochondrial gene CO1. To this end, 11 µl PCR reactions were prepared using Promega Hot Start GoTaq reagents including 5 µl Hot Start GoTAQ GREEN (Promega™), 4 µl of sterile distilled water (MilliQ Water), 0.5 µl of each primer and 1 µl of DNA template. Prepared PCR reaction samples were run in either a Bio-Rad®T100™ or Applied Biosystem® Veriti® 96-well thermo cycles. For the DNA quality PCR, primers LCO (forward): 5' GGT CAA CAA ATC ATA AAG ATA TTG G 3'; and HCO (reverse): 5' TAA ACT TCA GGG TGA CCA AAA AAT CA 3' were used, with 1 cycle of 2 minutes at 94˚C, then 35 cycles of 15s at 93˚C, 60s at 47˚C and 60s at

72˚C, followed by 1 cycle of 60s at 10˚C. All PCR assays were conducted alongside contemporaneous known positive and negative (blank) templates. Amplicons were visualised on 1.5% agarose gels containing 2 µl of Ethidium Bromide, alongside Bioline HyperLadder ^{TM} I size standard (1 kb). Gels were prepared using 1.5 g of agar and 100 ml of buffer TAE (Tris-acetate-EDTA; 1L of 50x TAE contains: 242 g of Tris Base, 75.1 ml of Glacial Acetic Acid, 200 ml of pH 8.0 EDTA and approximately 750 ml of RO Water). After electrophoresis, gels were visualised and photographed using UV-B light and the presence of absence of amplicon scored.

Templates that failed the QC were discarded from further analysis. The DNA templates passing QC were then tested for *Spiroplasma*, *Rickettsia* and *Wolbachia*, using primer combinations and PCR conditions given in Table 2.4. As before, all PCR assays were conducted alongside contemporaneous known positive and negative (blank) templates.

Positive PCR assay samples were sequenced for 1-5 individuals for each location for each symbiont infection to ensure these represented strains identified previously as male-killing bacteria. First, PCR product was cleaned of unincorporated nucleotides and oligonucleotide primer. To this end, 5 µl of PCR product was mixed with 0.2 µl shrimp alkaline phosphatase (BioLabs), 0.05 µl Exonuclease I (BioLabs), 1 µl 10 x SAP Reaction Buffer (BioLabs) and water to 10 µl. This was incubated at 37˚C for 45 minutes before killing enzyme activity by placing at 70˚C for 15 minutes. 1 µl of this product was used as a template in a 10 µl total volume with 0.18 µl BigDye 3.1 Terminator (Applied Biosystem®), 0.32 µl of one of the primers, 1 µl 5 x Reaction Buffer (BioLabs) and 7.5 µl water. This was subject to cycle sequencing program (25 cycles of 10s at 96˚C, 5s at 50˚C and 4 minutes at 60˚C, followed by incubation at 8˚C), and the product was then precipitated with 1.5 µl of 3M Sodium acetate (NaOAc) pH 4.6 and 31.25 µl of 100% Ethanol and centrifuged for 1400-2000 x *g** for 45 minutes. Then the pellet was cleaned by adding 150 µl of 70% Ethanol and centrifugation for 1400-2000 x *g** for 10 minutes. Final cleaned products were combined with 10 µl Hi-Di formamide (Applied Biosystem®) and then loaded onto an ABI 3730 automated sequencer.

DNA sequences obtained were first assessed for quality by eye on BioEdit™ and sequences similar to those on Genbank assessed examined through BLAST (Basic Local Alignment Search Tool) analysis on NCBI. The top hit was checked against the previously acquired sequences from ladybird symbionts. Expected hits were: *Spiroplasma* accession number AJ006775, Hurst *et al.,* 1999a; *Wolbachia* strain Y AJ130714 and *Wolbachia* strain Z AJ130715, Hurst *et al.,* 1999b; *Rickettsia* AJ269517, Hurst *et al.,* 1999a; Schulenburg *et al*., 2001b.

Table 2.4: Primer combinations and PCR conditions for molecular assays to test the presence of male-killing bacteria. All PCR cycles involved an initial melt phase of 2 minutes at 94˚C, and a final extension of 10 minutes at 72˚C (one exception for *Spiroplasma* final extension: 20 minutes at 72 ˚C).

Male-killing bacteria	Primers	Primer sequence	Gene region	PCR conditions	Expected amplicon size	Reference
Spiroplasma	HaIn-1 (forward)	5’ GCT CAA CCC CTA ACC GCC 3’	16S rRNA gene of *Spiroplasma*	35 cycles of 15s at 94˚C 60s at 55˚C 30s at 72˚C	429-bp	Hurst *et al.*, 1999a
	MGSO (reverse)	5’ TGC ACC ATC TGT CAC TCT GTT AAC CTC 3’				
Rickettsia	R1 (forward)	5’ GCT CTT GCA ACT TCT ATG TT 3’	17 kDa outer-membrane protein gene common to members of the *typhi* group of the genus *Rickettsia*	35 cycles of 20s at 95˚C 60s at 60˚C 60s at 72˚C	434-bp	Werren *et al.*, 1994
	R2 (reverse)	5’ CAT TGT TCG TCA GGT TGG CG 3’				
Wolbachia	wsp 81F (forward)	5’ TGG TCC AAT AAG TGA TGA AGA AAC 3’	wsp gene coding for an outer membrane protein of *Wolbachia*	30 cycles of 16s at 93˚C 60s at 55˚C 50s at 72˚C	610-bp	Braig *et al.*, 1998; Zhou *et al.*, 1998
	wsp 691R (reverse)	5’ AAA AAT TAA ACG CTA CTC CA 3’				

2.2.3.3 Analysis of variation in symbiont presence between samples

Consistency in the frequency of the dominant *Spiroplasma* infection was tested using a General Linear Model with binomial errors implemented in R software. Within this model the impact of sample location, time of sampling and the interaction between sample location and time of sampling, were investigated as sources of variation in the number of symbiont positive/negative individuals in a sample (response variable). This analysis covered the three years in which samples were collected over the majority of populations: 2001, 2011, and 2012.

2.3 Results

2.3.1 Mite incidence

Mites were recorded as present where more than one individual in a sample carried infection (this was to differentiate an established infection from ones associated with occasional migrants). Under this criterion, mite incidence showed a highly reproducible pattern of space, with data from 2011-2013 mirroring that collected 2000-2002 (Figure 2.4). In brief, the mite was present in Malmö, Gävle and Stockholm in all samples, but absent in Nässjö, Östersund, Ljusdal and Vilhelmina. Mite prevalence in the samples obtained in each year is given in the appendix, Table A2.1.

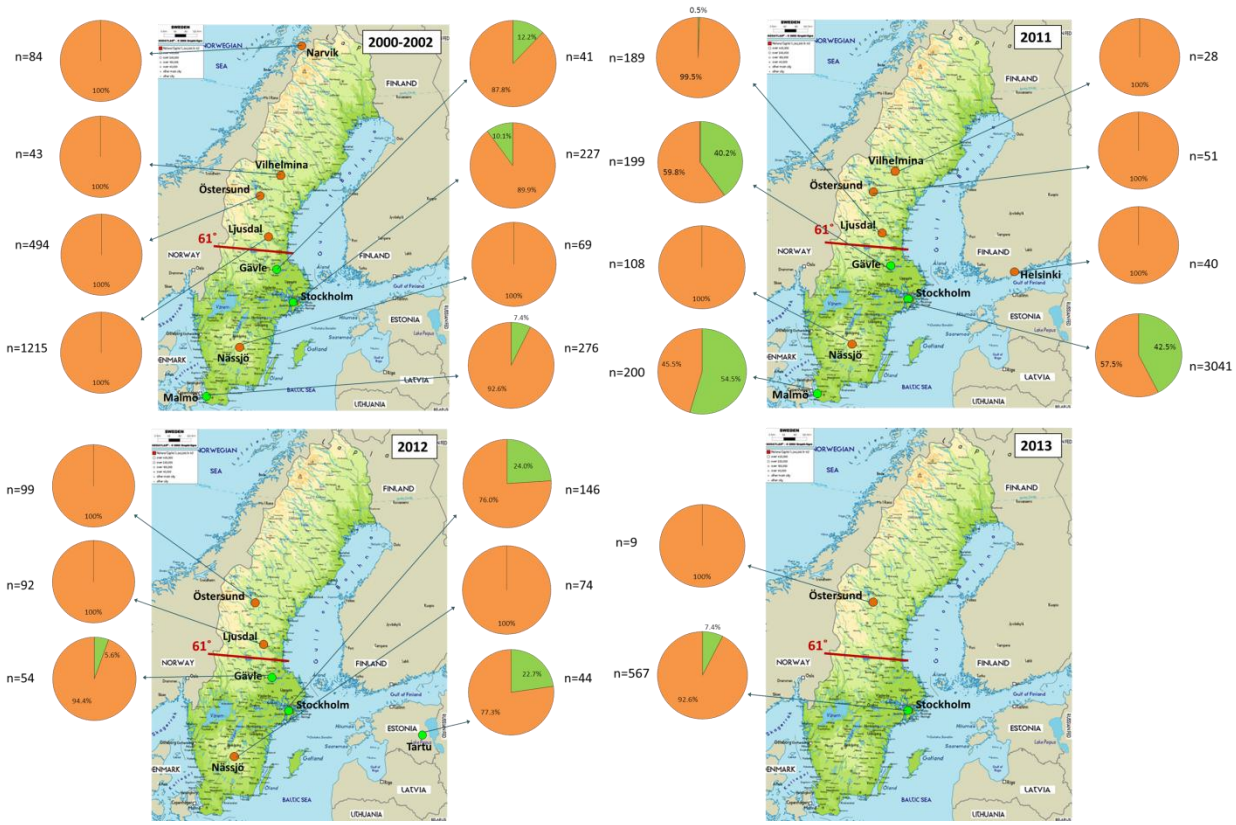


Figure 2.4: The presence and prevalence of mite *Coccipolipus hippodamiae* in Sweden over time (2000-2002 Tinsley's data; 2011-2013 data from this study). Pie charts represent proportion of collected ladybirds infected with mites. ● ladybird infected with mites; ● ladybirds not infected with mites. (Map source: www.in-sweden.co.uk/physical-map-of-sweden.html).

2.3.2 Prevalence and incidence of heritable male-killers

Symbionts previously identified as male-killing bacteria were identified across the species range (Figure 2.5). Symbiont infection was least common in northern populations in each sampling period, indicating a general pattern that male-killing symbionts were at higher prevalence in the south than the north.

The dominant male-killer observed was *Spiroplasma*. The higher prevalence of *Spiroplasma* in southern populations compared to northern, observed by Tinsley (2003) was also observed in the samples collected between 2011 and 2013 (Figure 2.6). Model analysis (Table 2.5) indicated samples were significantly heterogeneous

for *Spiroplasma* frequency. The location factor 'Site' was the largest contributor to model deviance. An interaction between 'Site' and 'Time' was the second largest contributor, indicating that some sites varied more over time than others. 'Time' itself contributed only a small element of deviance, reflecting changes in frequency occurring across the species range were relatively unimportant sources of heterogeneity.

Table 2.5: GLM model investigating the impact of sample location, time of collection and the interaction between sample location and time of collection on the presence of male-killing bacteria and their prevalence.

Factor	Df	Deviance Resid.	*P* (>Chi)
Location	6	205.838	$< 2.2e^{-16}$
Time	2	15.073	0.0005332
Location x Time	10	71.081	$2.741e^{-11}$

Variation over sites was persistent, and followed a pattern of high prevalence in the south of Sweden and low prevalence in Ljusdal, Östersund, and Vilhelmina. Time of collection contributed only minor heterogeneity when considered in terms of the whole species range, with the 2011 collection showing marginally significant deviation from others (*P*=0.042). Particular locations in which time of collection was associated with variation in prevalence were identified (the interaction term). Changes of large magnitude were observed in the Ljusdal sample, with samples of 2011 and 2012 both distinct from the 2001 sample (*P*=0.00119, *P*= $9.84e^{-07}$ respectively). In this population, *Spiroplasma* was rare in 2001-2002 (8/75 female beetles) but became common in 2011 (48/170) and 2012 (47/63). This site thus shifted from having a classic 'northern' low prevalence pattern of *Spiroplasma* infection in 2001-2002 to having a high prevalence 'southern' pattern in 2011 and 2012.

Adalia bipunctata has also been identified as carrying two other male-killer infections, a *Rickettsia* and *Wolbachia*. Of these, *Rickettsia* was recorded in the 2001/02 surveys, but *Wolbachia* was not detected. *Rickettsia* was again observed at

modest prevalence in the 2011 and 2012 surveys (Figure 2.7). However, 2011 and 2012 samples were distinct from that of 2002 in carrying *Wolbachia* (Figure 2.8). *Wolbachia* infection was rare (<10% prevalence in all cases), but pervasive, with records in the five most southern populations, but absence from Östersund and Vilhelmina. BLAST analysis indicated the *Wolbachia* strains found corresponded to both strain Y and strain Z described in Majerus *et al.* (2000).

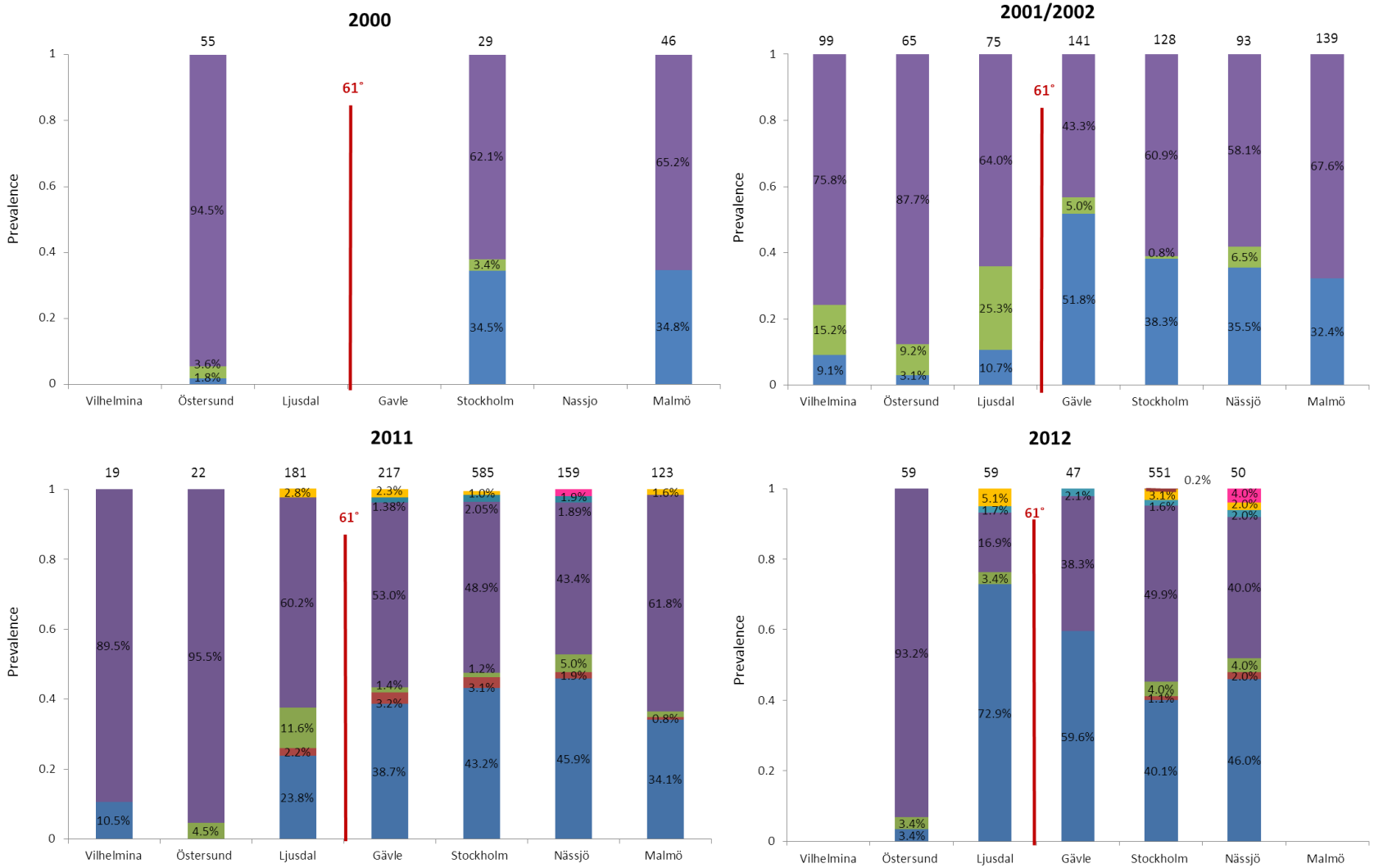


Figure 2.5: The prevalence of *Spiroplasma* (S), *Rickettsia* (R) and *Wolbachia* (W) in Scandinavia over time (2000-2002 Tinsley (2003) and 2011-2012 this study). Colour identification: ●*Spiroplasma*; ● *Rickettsia;* ● *Wolbachia*; ● Uninfected; ● Coinfection S-R; ● Coinfection S-W; ● Coinfection R-W; ● Coinfection S-R-W.

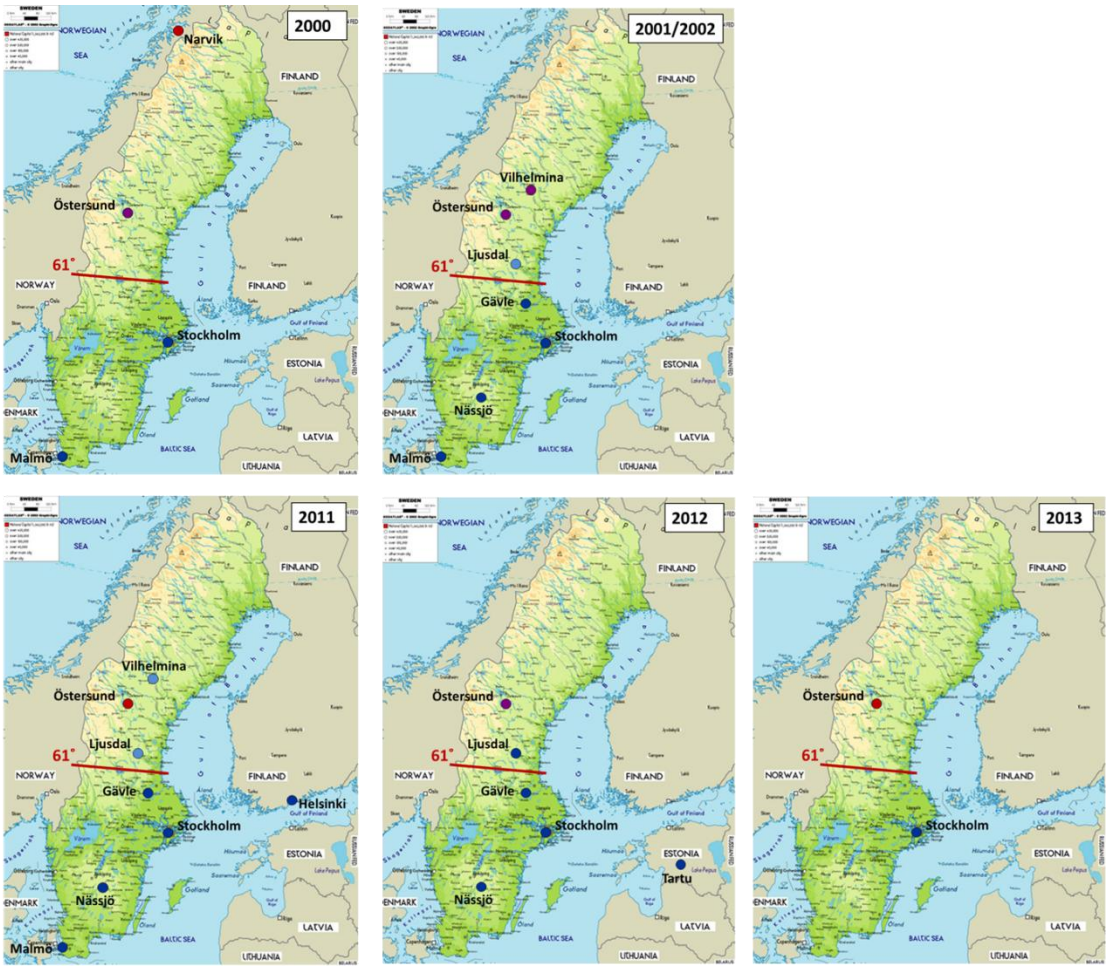


Figure 2.6: The occurence of *Spiroplasma* male-killing bacteria in Scandinavian countries over time. Sampling regime by colour: ● *Spiroplasma* prevalence was higher than 30%; ● *Spiroplasma* prevalence between 10-30%; ● prevalence of *Spiroplasma* reached up to 10% of females in *Adalia*; ● absence of *Spiroplasma* (Map source: www.in-sweden.co.uk/physical-map-of-sweden.html).

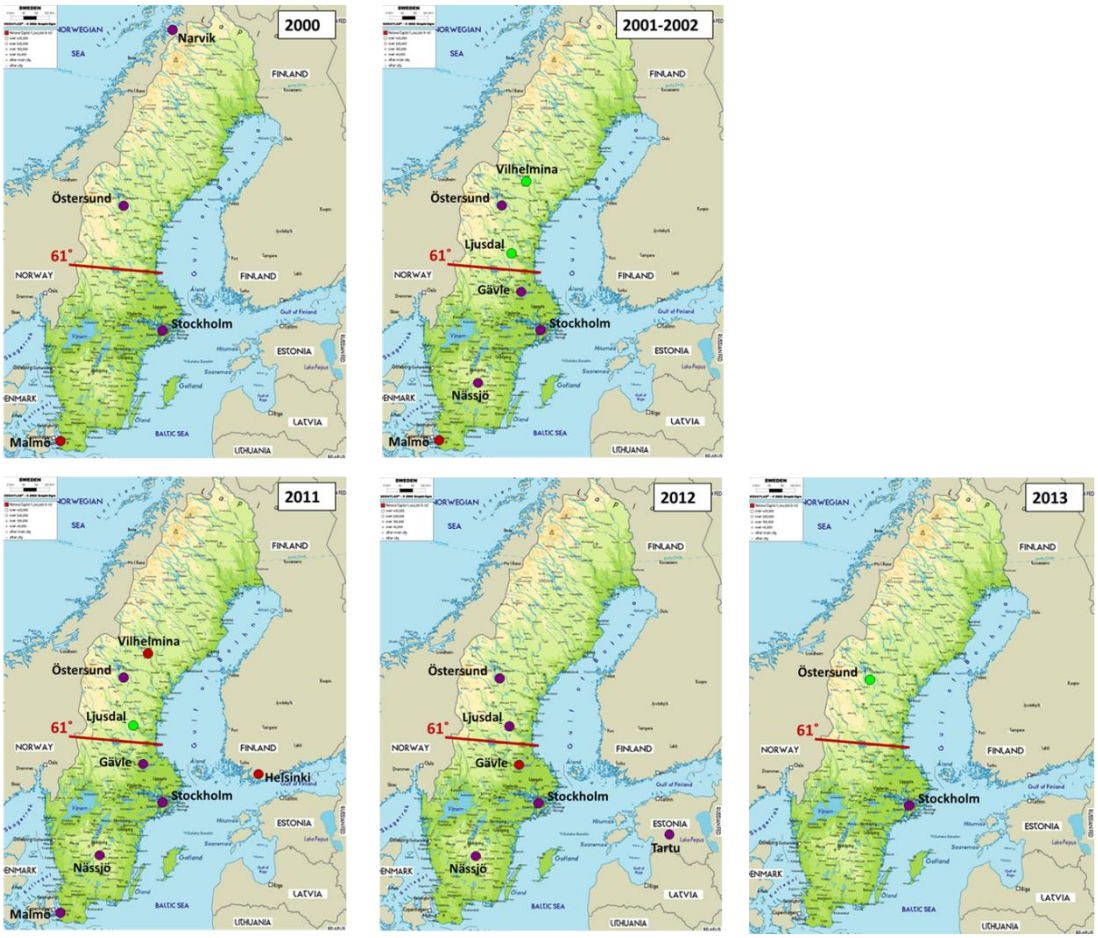


Figure 2.7: The occurence of *Rickettsia* male-killing bacteria in Scandinavian countries over time. Sampling regime by colour: ● *Rickettsia* prevalence was higher than 30%; ● *Rickettsia* prevalence between 10-30%; ● prevalence of *Rickettsia* reached up to 10% of females in *Adalia* populations; ● absence of *Rickettsia* (Map source: www.in-sweden.co.uk/physical-map-of-sweden.html).

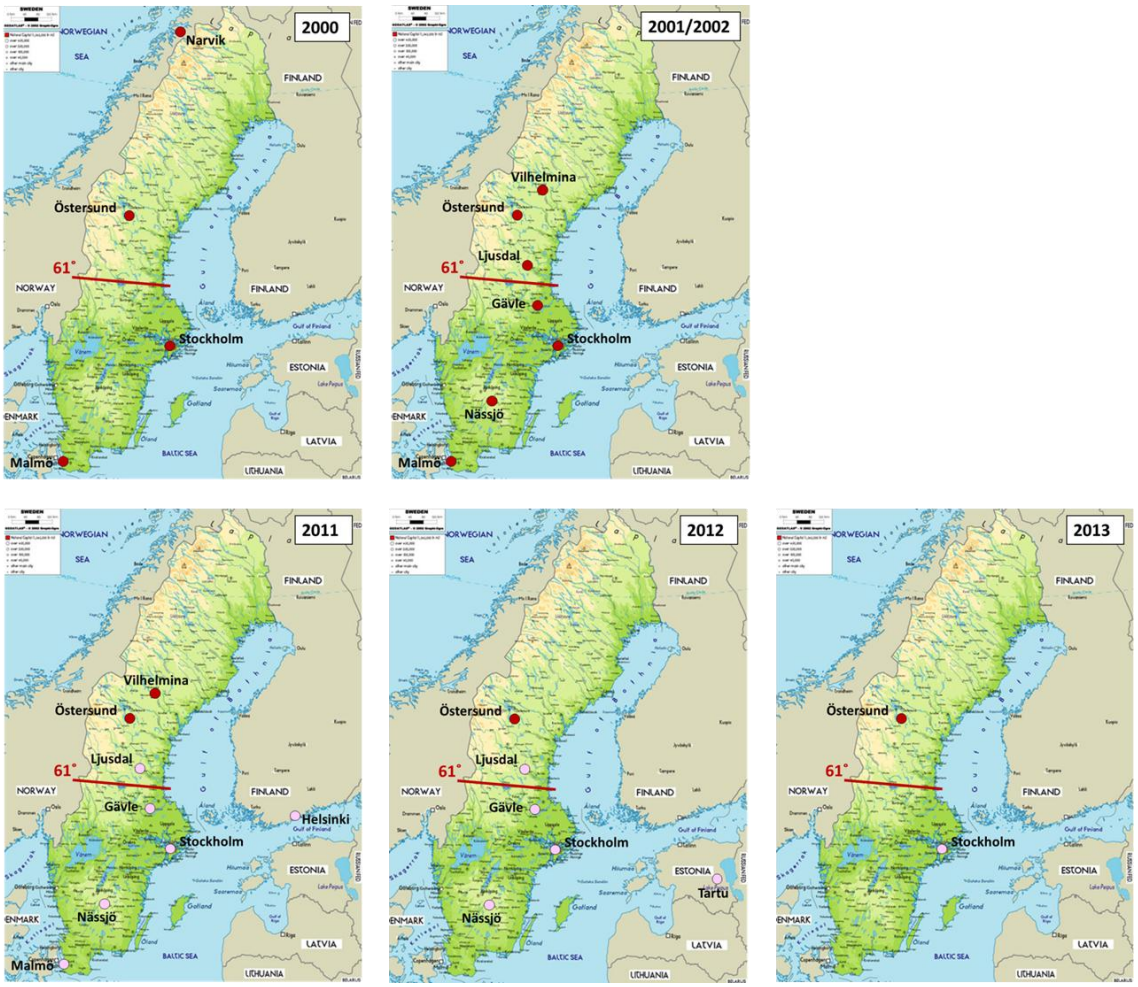


Figure 2.8: The occurence of *Wolbachia* male-killing bacteria in Scandinavian countries over time. Sampling regime by colour: ● *Wolbachia* prevalence was higher than 30%; ● *Wolbachia* prevalence between 10-30%; ● prevalence of *Wolbachia* reached up to 10% of females in *Adalia* populations; ● absence of *Wolbachia* (Map source: www.in-sweden.co.uk/physical-map-of-sweden.html).

2.4 Discussion

This study showed that there is a strong geographic boundary for mite presence with its absence in the north and presence in the south of Sweden (except one location, Nässjö). The distribution of mite infection in ladybird populations collected in this study precisely mirrored that recorded in the period 2000-2002. Thus, it is clear that the spatial variation in mite incidence is stable in Sweden, at least over broad geographic scales.

This data presents a slight contrast to non-Scandinavian European populations where previous surveys have indicated some populations to be infected whenever sampled, some uninfected whenever sampled, and some varying in the presence of infection, for instance in Bayreuth (Germany) and in St Petersburg (Russia). These later populations were at the edge of the mite range (Webberley *et al.,* 2006b). The collections made for this study contrast with this, and reflect stability of the northern boundary of mite incidence within Sweden.

It seems very unlikely that the lack of change in mite incidence is associated with lack of introduction of the mite to uninfected populations. Ladybirds are mobile and able to move easily between different sites, so that they can search for new food resources especially when aphids are limited (Brakefield, 1984; Webberley *et al.,* 2006a). Evidence from this study also indicates that the mite is introduced to the northern populations occasionally. During sampling, one ladybird collected in Ljusdal, a northern city where the mite was not regularly found, was recorded as carrying a mite infection (in a sample of 189 individuals). The absence of mites in the next generation indicates that the observation of one mite infected beetle can be best explained by arrival of an immigrant mite-infected ladybird from the south (where mite is common), into a northern population where mite is absent because conditions do not support its maintenance.

The consistency of mite incidence is thus not explicable through isolation and lack of introduction to the mite. Rather, it indicates an underlying ecological or biological driver that determines whether the mite can persist. Previous work has proposed

that phenology may determine mite incidence, through altering the rate at which members of different beetle generations meet and mate (Hurst *et al.*, 1995; Knell and Webberley, 2004). Alternatively, there may be biological factors, such as host resistance to infection or difference in host behaviour (e.g. mating rate) that determine mite incidence. These hypotheses are evaluated in Chapter 3.

Heritable male-killer prevalence and incidence was also broadly similar between the 2000-2002 and 2011-2013 samples. Three observations are notable. First, *Spiroplasma* remained the most dominant male-killing bacteria among collection sites. Second, *Spiroplasma* prevalence remains broadly stable since the 2002 survey, with high prevalence in the south and low prevalence in the north. On the other hand, a northward movement of *Spiroplasma* was noticed. In the 2002 survey, Ljusdal marked the most southerly location where *Spiroplasma* was rare/not found. In contrast, this symbiont was common in Ljusdal in the 2011 and 2012 collections (26.5% and 79.7% prevalence respectively). There is evidence of a rapid increase in frequency of *Spiroplasma* in this population over time.

It is not currently known what ecological and biological factors cause the boundary between southerly pattern of high *Spiroplasma* prevalence, and northerly pattern of low *Spiroplasma* prevalence. Northerly shifts are currently being observed in multiple species, associated with climate change, and it is tempting to suggest that this data represents a case of a symbiont undergoing a similar shift. This would suggest *Spiroplasma* incidence is ecologically driven, rather than being driven by biological features such as evolved host resistance to infection. The ecological drivers may act either on the benefit of male-killing, the cost of carrying infection, or the transmission efficiency of the symbiont.

a) Benefit of male-killing. Majerus and Majerus (2012) suggest that northerly absence of male-killers may be associated with a short, intense breeding season. The length and intensity of winter is such that ladybird breeding is delayed compared to southern populations, and is compressed into a short (one month) period. They propose that aphid food is superabundant during this compressed season (as natural enemies fail to regulate aphid population

growth). Because the benefit to male-killing derives from providing tolerance to low food during early larval stages, superabundance of food may reduce any benefit of male-killing activity, and thus be associated with lower symbiont prevalence, and at some point, failure to persist.

b) Cost of carrying infection. Symbionts impose a direct metabolic cost to their host, and may also provide ecologically contingent benefits. Some of these costs will appear most under stress. Northerly winters are both colder and longer than those in the south. Thus, should *Spiroplasma* have any impact on host mortality during the winter period, the costs could be felt more strongly in the north.

c) Transmission efficiency. A fraction of daughters of infected females do not inherit the infection. The transmission rate of an infection can be affected by environmental conditions. *Spiroplasma* in *Drosophila melanogaster* and *Drosophila hydei*, for instance, fail to be transmitted at temperatures below 18˚C, and this probably explains the absence of *Spiroplasma* from *D. melanogaster* populations outside the tropics (Anbutsu *et al.*, 2008; Osaka *et al.*, 2008). It is possible that cold curing exists for this infection, or that transmission is modulated in reproductive diapause (winter). Recent studies have revealed that prolonged presence of cold temperatures and/or longer diapause of a host reduce the vertical transmission efficiency of *Wolbachia* in *Nasonia vitripennis*, associated reduction in *Wolbachia* titre during prolonged diapause (Perrot-Minnot *et al.,* 1996).

To date, the information to support/reject these hypotheses is sparse. There is no information relevant to the food and transmission efficiency hypothesis. With respect to overwinter mortality, Tinsley (2003) noted no impact of *Spiroplasma* on overwinter survival in samples kept at 0.15˚C. However, this regime as more benevolent than a Swedish winter, as it does not include freeze tolerance.

The hypotheses thus require more detailed testing. The importance of food supply would be implied by lower first instar larval mortality through starvation in northern climes. The role of overwinter temperature as a source of stress under which costs of infection are exacerbated can be tested through placing ladybirds in field overwintering boxes and examining the survival of infected vs uninfected females. The role of winter in affecting transmission efficiency can be examined from the infected survivors of such an experiment.

Aside the northward shift in *Spiroplasma* prevalence, the survey revealed little change in the low prevalence of *Rickettsia* male-killers across Sweden, and the emergence of *Wolbachia* symbiont infections in a small number of beetles. Both of these symbionts are rare and thus not important drivers of population sex ratio/ladybird biology. Further, PCR assays suggest the presence of co-infected ladybirds – ones with *Spiroplasma* and either *Rickettsia* or *Wolbachia*. However, this is not discussed further as their sporadic nature makes it hard to assess if they are simply a product of false positive PCR assays or occasional contaminants.

The presence of multiple symbiont infections in Sweden is, in contrast, clear. It repeats the pattern of Tinsley (2003) for *Rickettsia*/*Spiroplasma*, and suggests the additional emergence of *Wolbachia* symbionts. The pattern of infection with multiple symbionts is also observed in Moscow, where all three symbiont infections are recorded (Majerus *et al.*, 2000). Multiple infections with male-killers is also seen in other species, for instance in the Tanzanian population of the butterfly *Acraea encedon* in which there are two strains of male-killing *Wolbachia* (Jiggins, 2001). These findings are counter to simple theory which predicts the co-existence of several male-killing bacteria in one population is unstable at equilibrium (Randerson *et al.,* 2000).

In parallel with theories for the maintenance of genetic variation, the most obvious possibility for the maintenance of multiple male-killers is that different symbionts are favoured in different geographical locations, and that multiple symbiont infections exist locally due to gene flow. Geographical variation in persistence of symbionts is indeed evident in this study: *Spiroplasma* bacteria appear less able to

persist in the north. Thus, spatial heterogeneity with a low level of insect host movement remains a good hypothesis for the co-occurrence of symbionts within a population. In this model, *Spiroplasma* infection is maintained in the north from movement of individuals from southern Sweden, *Rickettsia* infection in the south is maintained from movement from either the north, or from Denmark (where *Rickettsia* infection alone is known: Hurst *et al.,* 1999b). The source of *Wolbachia* infection remains unclear. This symbiont infection has been recorded in Moscow and Tomsk, but has not been apparent in surveys in Poland or Denmark, nor in samples from St Petersburg (Russia). Samples from Helsinki, Finland and Tartu, Estonia also revealed only *Spiroplasma* infection.

In summary, this chapter has revealed consistency of mite presence in *Adalia* in Sweden, and this pattern is investigated further in the next chapter. The pattern of symbiont presence shows some consistent features (southerly presence of high prevalence *Spiroplasma*, northerly absence; *Rickettsia* infection persistent but rare in all populations), and some changes (presence of *Spiroplasma* in Ljusdal with rapid increase in frequency; presence of *Wolbachia* in 2011-2012 samples). The factors determining male-killer prevalence and incidence remain enigmatic, and are left for further study.

Chapter 3: Host phenology limits the incidence of an insect sexually transmitted infection

1. Whilst many species of insect are promiscuous, sexually transmitted infections are not present in all promiscuous species of insect. Notwithstanding this, the ecological and biological factors that permit STI maintenance in insects have received little attention.
2. In this chapter, the factors underlying the presence/absence of the sexually transmitted mite, *Coccipolipus hippodamiae* in Swedish populations of its two-spot ladybird host, *A. bipunctata*, are investigated. As noted previously, the mite was absent north of 61˚N, but present south of 61˚N, except in the higher altitude population of Nässjö.
3. I evaluated two explanations for the absence of the STI in some populations. The first hypothesis was that ladybirds in the populations where the mite was absent were biologically not competent to carry/transmit infection. The second hypothesis was that the phenology of the host species interfered with transmission of the mite between generations of the host, and prevented mite persistence.
4. Laboratory experiments demonstrated ladybirds in the north of Sweden could be infected by, and transmit STI infection onward.
5. In contrast, there was evidence that phenological changes were associated with interruption of mite transmission between cohorts. Ladybirds collected in August 2012 from two northern sites where the mite was not present contained, 2012 cohort individuals alone, and none of these females had mated indicating there was no sexual contact with the previous generation. In contrast, an August 2012 collection from the two sites where mite infection is persistent revealed both old and new generation ladybirds, and that some of the new cohort female ladybirds had mated.
6. I thus conclude that host phenology may determine the incidence of STIs in temperate insect species.

This chapter has been accepted for publication in modified form as:

Pastok *et al.* "The role of host phenology in determining the incidence of an insect sexually transmitted infection." *Oikos*, accepted.

3.1 Introduction

A large number of animal species carry sexually transmitted infections (STI), pathogens and parasites which are primarily transmitted during copulation. To date, over 60% of records of STIs come from arthropods. In contrast to STIs in mammals, the infectious agents are largely fungi (such as the Laboulbenialies) or macroparasites, for instance mites and nematodes (Knell and Webberley, 2004). Infection by an STI commonly has a significant impact on an individual's fitness. This impact can be severe, because STIs are very often highly pathogenic and can reduce host fitness, for example by sterilizing their host (Hurst *et al.*, 1995). Because the effects of STIs reduce host fitness, they represent important drivers of host ecology, and may also be important contributors to the evolution of host mating behaviour (Hurst *et al.,* 1995; Lockhart *et al.,* 1996; Webberley *et al.,* 2002; Knell and Webberley, 2004).

The dynamics of STIs are very often related to host mating system, and the majority of research has concentrated on how mating systems are likely to impact on STI epidemiology. However the age structure in STI systems can also have an important effect, especially in populations where reproduction is strongly seasonal and the presence of separate generations is observed. The simplest case represents two cohorts – a parental generation and their offspring in one year. Although this pattern is common in insects in mild environmental conditions, it could be also observed in insects from tropical habitats with 'generation cycles' (Knell, 1998).

As Knell and Webberley (2004) noted, the presence of cohort structure is very important for STI dynamics, which then becomes a product of both within and between cohort transmission events. The cohort structure may at its extreme provide a hard barrier to STI persistence. Sexually transmitted infections are only diseases of adults therefore cohorts of adults must overlap so the parasite can be transmitted between them and persist in the host population. An STI would disappear from a host population where there was a 'generation gap': periods where no adult individuals are present, or where adults from one cohort have an obligatory diapause before commencing mating activity. Knell and Webberley

(2004) also outlined the other conditions which are required for species to maintain an STI. These are promiscuousness of host species and a parasite life cycle. Firstly a host must be promiscuous so individuals mate with several different partners over their life history and consequently they have more chances to acquire and transmit a parasite. Secondly a parasite life cycle has to be completed before host sexual activity is finished. This requires that the host is relatively long lived, and has sexual contact spread over its life history rather than over a single short period. To date no study has investigated the degree to which the conditions defined by Knell and Webberley (2004) account for STI presence/absence in nature.

The ladybird *Adalia bipunctata* and the mite *Coccipolipus hippodamiae* provides an excellent system to test these hypotheses because the basic biology of interaction and the transmission are established (Hurst *et al.,* 1995). There is also a good understanding of mite epidemiology within a ladybird generation (Webberley *et al.*, 2006a; Ryder *et al.*, 2013; Ryder *et al.*, 2014). Finally, geographical variation in the presence of the mite STI exists, which is currently not well understood (Webberley *et al.*, 2006b). As outlined previously, the mite infection was first recorded around twenty years ago in Europe (Hurst *et al.,* 1995) and this system presents a good test system, as there is geographical variation in the presence of the mite STI within *Adalia*.

In this chapter, the cause of mite presence/absence in populations in Sweden is evaluated. Surveys between 2000 and 2002 revealed the mite was present on *Adalia bipunctata* in three of four Swedish populations south of 61˚N: Gävle, Stockholm and Malmö but being absent from a population in Nässjö (57˚39'N, altitude 375 m). However the mite was absent in all three populations north of 61˚N (Vilhelmina, Östersund and Ljusdal) (Webberley *et al.*, 2006b). In Chapter 2, the consistency of mite presence/absence data over the last 10 years was noted, with the mite observed in southern lowland populations, but absent in southern highland populations, and in all populations north of 61˚N (Figure 2.4). Further, it was noted that occasional infected ladybirds were observed in northern populations, implying that mite absence is associated with failure of the infection to

persist rather than barriers to its introduction. Thus, there is a repeatable pattern of absence/presence which must have a biological or ecological explanation.

Biological differences that could drive mite absence include failure of mites to be transmitted to ladybirds, failure to develop infections through to maturity, and evolved differences in promiscuity. Ecological explanations for the lack of persistence of the mite in northern populations centre on ladybird phenology and the requirement for 'overlap of generations' for mite persistence. Past studies of similar systems, have proposed these 'generation gaps', in which overwintered and new cohort beetles are separated in time, as a driver of STI absence (Hurst *et al.*, 1995; Seeman and Nahrung, 2004) but this hypothesis has yet to be tested.

Ladybirds emerge from overwintering in March-June, depending on latitude and altitude, and lay eggs (Figure 3.1). These eggs develop through four larval instars to a pupa, and finally an adult. Adult individuals then mature for one or more weeks before becoming reproductively active. As their adult parents begin to die after oviposition, an insufficient number of old generation adult ladybirds present alongside new cohort individuals could limit mite persistence. The hypothesis of phenological differences creating a hard stop is supported by observations of ladybird phenology from Tinsley (2003). He observed ladybird life stage presence in late spring/early summer in each of the years 2000, 2001 and 2002 across Sweden (for data, see Appendix A3.1, A3.2 and A3.3). At each location he visited, the presence of egg, larval and pupal stages was noted on a South-North transect where the northern populations were visited a few days after Southern ones (Tinsley, 2003). These observations indicate ladybird reproduction starts up to one month later in northern populations than southern ones, and that new generation beetles therefore emerge later in the year. In particular, I examined whether phenological variation could create a 'hard stop' in northern populations, where no transmission between generations was possible because old cohort individuals had died off before sexual activity of the next cohort had commenced (Figure 3.2).

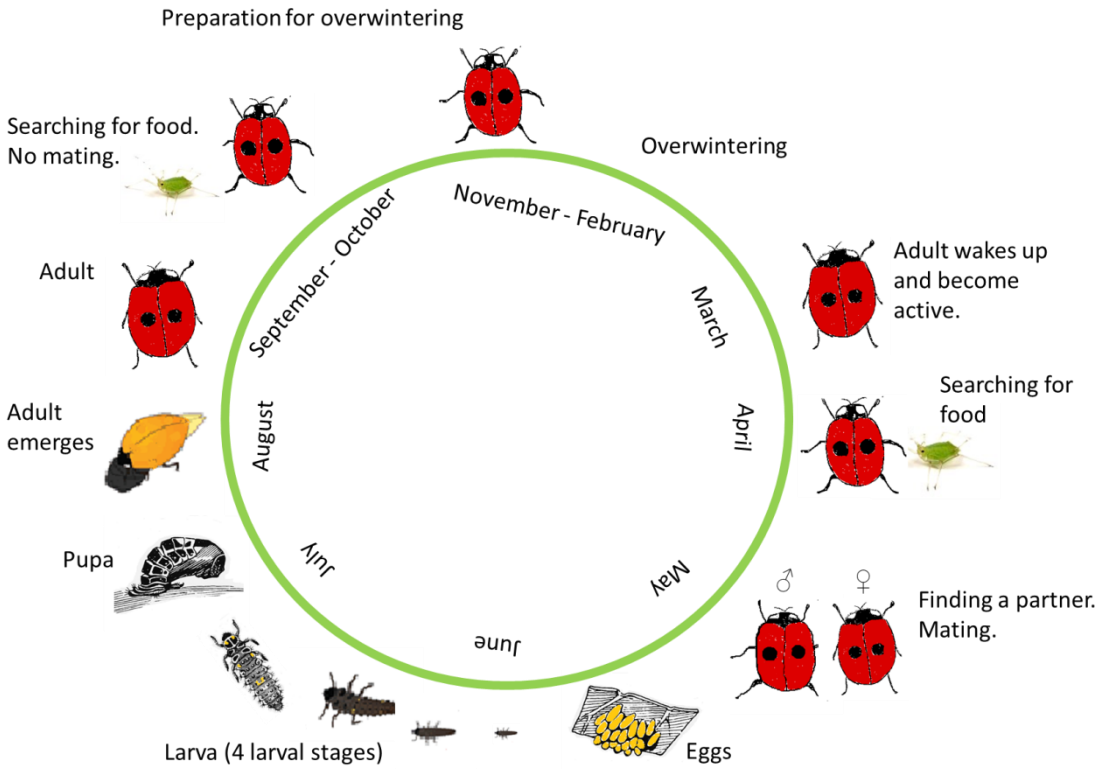


Figure 3.1: *Adalia bipunctata* life cycle as typically observed in the Stockholm population. Timings are approximate and vary between years, with new generation ladybirds emerging in July-August.

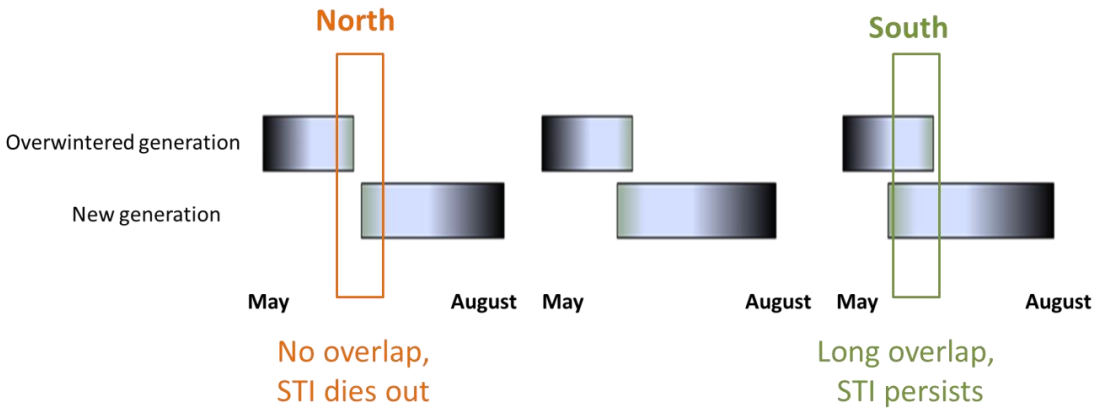


Figure 3.2: Pictorial representation of the potential phenology of *A. bipunctata* in Sweden. For each location (north > 61˚N, south < 59˚S), the presence of mature adult *A. bipunctata* is given through a season, with the overwintered generation presented on the top bar, and the adult progeny of the next cohort below. Dark shading represents larger population size.

In this chapter, the role of biological differences and phenological changes as forces underlying mite absence in northern populations is evaluated. The former was evaluated using laboratory experiments that a) examined the ability of the mite to transmit to, and onward from, ladybirds in northern populations; b) compared promiscuity levels of *A. bipunctata* in a common garden laboratory environment. The role of phenology was ascertained through field collections, combined with laboratory tests that indicated prior mating history, with the hypothesis that new cohort ladybirds collected in August in populations without the mite would be virgin, whereas some new cohort individuals collected at the same time in populations with the mite would have mated, thus exposing them to the STI.

3.2 Material and Methods

3.2.1 Is the absence of the mite from the north associated with an inability of the parasite to grow and transmit on ladybirds from the north?

I tested the ability of ladybirds from populations that did not carry mites naturally to become infected with larval mites, develop infection and transmit the mite infection onward. To this end, *Adalia bipunctata* were collected using beating tray in Östersund and Ljusdal (where the mite is absent) and in Stockholm (where the mite is present) in early August 2012. Ladybirds were transported to the laboratory where they were sexed and scored for mite presence under a binocular microscope. Mites were identified as being present on ladybirds from Stockholm. All ladybirds collected in Sweden were kept in an incubator at 20˚C with a light regime of 20L:4D (20 hours of light and 4 hours of night when the temperature decreased to 10˚C) and they were housed in plastic 9 cm diameter Petri dishes (a tri-vent Petri dish from Sarstedt™). Each Petri dish was labelled individually. All ladybirds were fed aphids daily where available and artificial food on all days.

3.2.1.1 Initial transmission and development of infection

Thirteen uninfected ladybirds from Östersund (mites naturally absent), six uninfected ladybirds from Ljusdal (mites naturally absent) and 19 uninfected ladybirds from Stockholm (mites naturally present, control) were isolated individually in Petri dishes, kept in the incubator at 20˚C and fed aphids daily. These individuals were then paired with a mite infected partner (from Stockholm) and mating observed. The next day ladybirds were separated from their infectious partner and then checked for the presence of larval mites (successful initial transmission). Mite persistence and disease latent period (the time from initial infection to infectious larval forms being produced) was then measured. To this end, recipient ladybirds were checked for mite presence on the seventh day, fourteenth day, nineteenth day post infection, and then daily for the progress of mite infection (Figure 3.3).

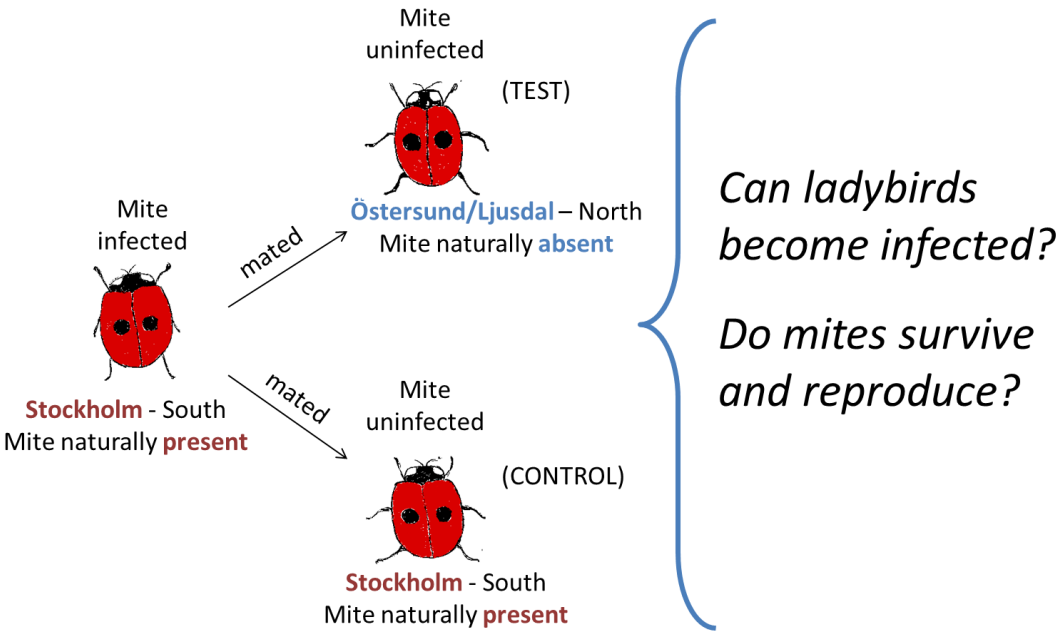


Figure 3.3: Experiment: Initial transmission and development of infection – testing if ladybirds from northern populations (where mite is naturally absent) become infected.

3.2.1.2 Onward transmission of infection

Where infection developed, the ability of the host to transmit mites onwards was tested. One week after the ladybirds above were first scored as carrying larval mites (the infectious stage), they were paired with an uninfected partner from the same population. Mating was observed as before, and the recipient checked for mite presence the next day. Development of infection was again ascertained by scoring mite presence on the seventh day, fourteenth day, and nineteenth day post exposure and then daily for the progress of mite infection (Figure 3.4).

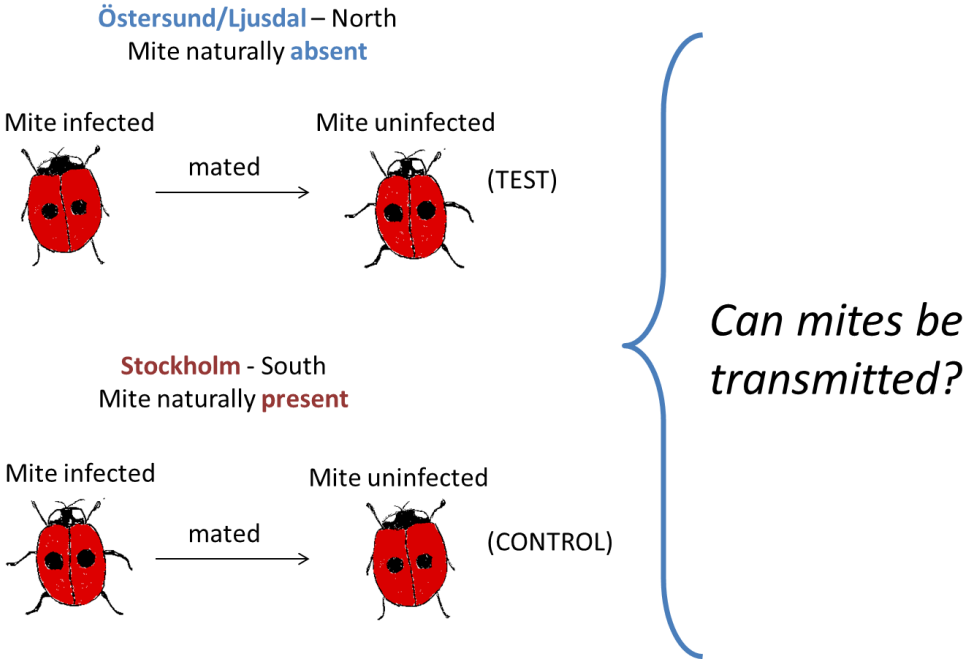


Figure 3.4: Onward transmission of infection – testing if northern infectious ladybird from the previous experiment can transmit mite to its partner.

3.2.2 Phenology of the host: sexual contact between generations and general observations on timing of reproduction

The quantity of sexual contact between cohorts was examined in detail through weekly sampling in Stockholm (mite present population) in May-August 2011. The detailed examination of overlap between generations in a mite present population was combined with a more focussed analysis of whether new cohort ladybirds made sexual contact with the overwintered cohort in three mite-free and two mite-present Swedish populations. This was completed by examining the state of these populations in early August 2012, enquiring a) whether new and old generation ladybirds co-occurred; b) if new cohort ladybirds had commenced sexual activity. These directed observations were then combined with more general phenological information regarding the timing of ladybird reproduction gained during survey work conducted in 2011.

3.2.2.1 Temporal sampling in Stockholm in 2011

The presence of overwintered and new generation ladybirds and the prevalence of the mite in Stockholm (mite present population) were recorded in weekly collections over the period of the 21st May-27th July 2011. Ladybirds were collected by beating from lime trees in city centre and suburban habitats, with aim of collecting 100 beetles per location, or if this number of beetles could not be obtained, that which could be found in one hour. Individuals were also noted as mating or single on collection. Collected ladybirds were sexed, scored for mite presence, and scored as to cohort through elytral colour. Cohort can be ascertained from the depth of elytral colour, with individuals belonging to an overwintered generation being deep red/black, in contrast to the orange/red of adults from the newly emerged generation (Majerus, 1994) (Figure 3.5).

3.2.2.2 Analysis of overlap between cohorts in mite absent/mite present populations

From the Stockholm data, it was clear that July and August represents the period when overwintered and new generation adult ladybirds may be present together, a necessary condition for transfer of the mite between generations. A break in the mite transmission cycle would occur when new cohort of ladybirds emerged after the death of overwintered cohort. This would be evidenced by the presence of virgin new cohort beetles in August with no overwintered beetles present. The co-occurence of new and overwintered cohort adult ladybirds, and the matedness of female new cohort ladybirds, was therefore examined in five populations in August 2012 spanning the region where the mite showed presence/absence differentiation: Östersund, Ljusdal (northern, both mite absent), Gävle, Stockholm (southern, both mite present) and Nässjö (southern, mite absent). Adult ladybirds were collected using a beating tray and by hand, and transported to the laboratory where they were scored for sex, mite presence and for cohort using the characteristic described above and on Figure 3.5. Alongside the collection of adult ladybirds, notes were made on the presence of other life history stages in the population, as an indicator of the timing of new cohort emergence.

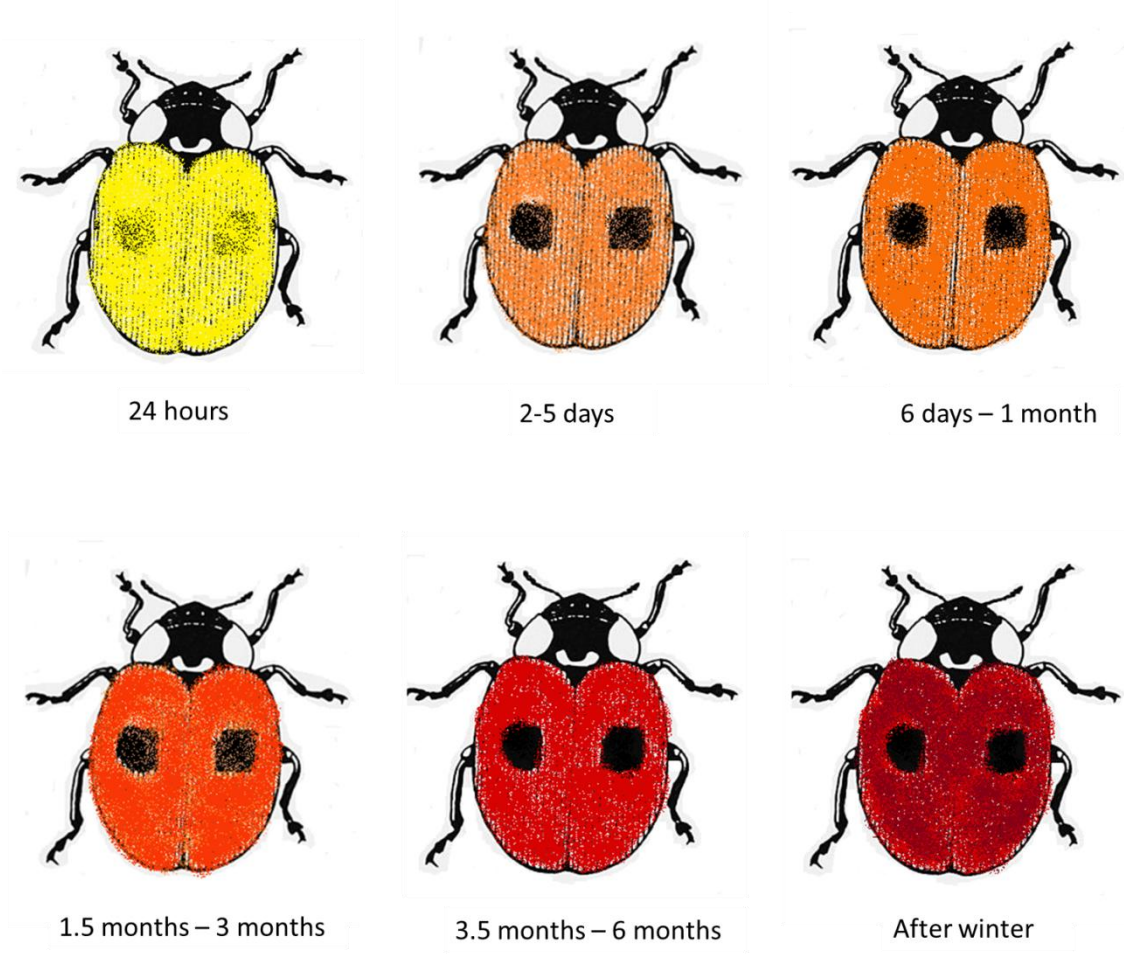


Figure 3.5: Pigment development in the two-spot ladybird *(Adalia bipunctata)*.

The presence or absence of previous female sexual activity of new cohort females from collected samples was then ascertained through examining their fertility. Virgin female *A. bipunctata* lay eggs at a low rate, such that lack of previous mating is indicated either by failure to lay eggs, or failure to lay viable eggs. To this end 25 new cohort ladybird females from each site were separated to individual Petri dishes. They were fed aphids daily. Eggs laid by females were collected and kept in the incubator at 24˚C. After 3-5 days eggs were checked if they hatched (indicating prior mating) or became shrivelled (indicating no sperm stored). For females that did not lay fertile eggs during this process, we then determined whether they were fertile by permitting them to mate to males from their own population, collecting eggs and assessing their fertility (Figure 3.6).

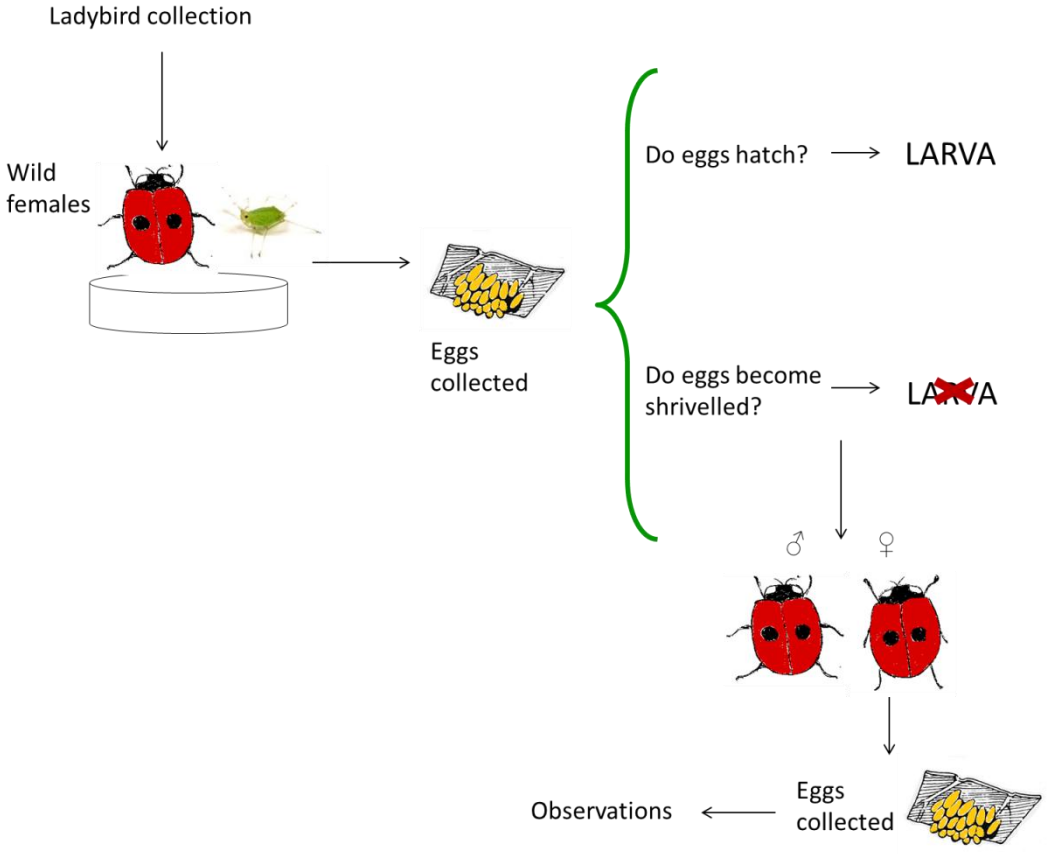


Figure 3.6: Method for examination of the fertility of field collected females. Oviposition is supported through *ad libitum* food. Eggs laid are then examined for viability. Females that do not lay eggs, or lay inviable eggs, have fertility tested by pairing with a male, and egg viability retested.

3.2.3 General phenological information on the timing of ladybird reproduction

Notes were made on the absence/presence of eggs, larvae and pupae on trees in May-July in 2011 whilst collecting ladybirds for the mite survey above. From this recorded data, the time at which oviposition began in the population was estimated as the earliest date of a) direct observation of eggs; b) observation of other life history phases, and then estimating the date at which the eggs from which these derived would have been laid (laboratory measurement estimates that egg-pupal development takes 21 days at 20˚C, including 6 hour night time temperature of 12˚C).

3.3 Results

3.3.1 Is the absence of the mite from the north associated with an inability of the parasite to grow and transmit on ladybirds from the north?

Northern ladybirds from Östersund and Ljusdal were successfully infected after mating to infectious partners derived from Stockholm. Infection was acquired in 14 of 15 cases where the recipient ladybird was from the north, compared to 15 of 16 control cases (recipient ladybird from the Stockholm) (Table 3.1). Binomial GLM revealed no interaction between location of recipient ladybird and sex of recipient ladybird. The model with the interaction term removed showed that there was no evidence for an effect of either location of origin or sex of those recipient ladybirds, on the chance of acquiring mite infection (Table 3.2).

Table 3.1: The proportion of ladybirds from north/south which acquired mites after mating with a mite infectious partner partitioned by sex and origin of recipient.

Origin of recipient beetle	Sex of recipient beetle	*P* (infected through mating) (*N*)
Stockholm	Male	0.89 (9)
Stockholm	Female	1.0 (7)
Ljusdal/Östersund	Male	0.86 (7)
Ljusdal/Östersund	Female	1.0 (8)

Table 3.2: Binomial GLM analysis of impact of location and sex of beetle origin on chance of acquiring infection from an infected partner during copulation.

	df	Deviance	AIC	LRT	*P* (>Chi)
None		0.000	9.7343		
Location	1	0.03604	7.7704	0.03604	0.84944
Sex	1	2.80863	10.5430	2.80863	0.09376

I then examined the ability of the mite infection to develop on the northern (novel) hosts. Eleven ladybirds out of 14 became infectious with the mite by day 17, two hosts died with a live mite infection, and one host recovered (mite infection lost). This compared to 15 control recipients, where 10 hosts became infectious, five hosts died with a live mite infection, and no host recovered (Table 3.3). Analysis indicated there was no evidence to reject the null hypothesis of no impact of ladybird population source on the chance of recovery following initial infection (Table 3.4, 3.5 and Table 3.6; the interaction term was non-significant and was removed from the analyses). Data on latent period was not collected on a daily basis and the formal analyses were not done. However, the data do not provide obvious signs of a difference in latent period between ladybird hosts from Stockholm and Östersund/Ljusdal (Table 3.7).

Table 3.3: The fate of mite infection on ladybirds from north/south, partitioned by sex of recipient host.

Origin of recipient beetle	Sex of recipient beetle	Number infected	Fate of infection		
			Host recovered	Host died before becoming infectious	Host became infectious
Stockholm	Male	8	0	2	6
Stockholm	Female	7	0	3	4
Ljusdal/Östersund	Male	6	1	0	5
Ljusdal/Östersund	Female	8	0	2	6

Table 3.4: Binomial GLM analysis of impact of location and sex of beetle origin on mite development.

	df	Deviance	AIC	LRT	*P* (>Chi)
None		0.00377	15.206		
Location	1	1.07081	14.273	1.06704	0.3016
Sex	1	0.29619	13.498	0.29242	0.5887

Table 3.5: Binomial GLM analysis of impact of location and sex of beetle origin on host survival despite mite infection.

	df	Deviance	AIC	LRT	*P* (>Chi)
None		1.4873	14.866		
Location	1	4.0922	15.471	2.6049	0.1065
Sex	1	2.6379	14.017	1.1506	0.2834

Table 3.6: Binomial GLM analysis of impact of location and sex of ladybird origin on host recovery from mite infection.

	df	Deviance	AIC	LRT	*P* (>Chi)
None		0.0000	7.8232		
Location	1	1.9412	7.7644	1.9412	0.1635
Sex	1	1.7982	7.6214	1.7982	0.1799

Table 3.7: Estimated latent period of mite infection on ladybirds from north/south, partitioned by sex of recipient host.

Origin of recipient beetle	Sex of recipient beetle	Number	Latent period		
			≤14 days	15-17 days	>17 days
Stockholm	Male	6	5	1	0
Stockholm	Female	4	2	2	0
Ljusdal/Östersund	Male	5	3	1	1
Ljusdal/Östersund	Female	6	4	2	0

Finally, I checked if mites can be transmitted onward from northern ladybird hosts. I observed onward transmission for four of five experimental matings to uninfected northern ladybirds, and six of six control matings (Stockholm source and recipient). There was no evidence origin of recipient ladybird affected transmission of infection (Table 3.8; Table 3.9).

Table 3.8: The proportion of ladybirds from the north and the south that transferred mites onwards during copulation with an uninfected partner.

Origin of recipient beetle	Sex of recipient beetle	*P* (ladybirds infected through mating) (*N*)
Stockholm	Male	1 (4)
Stockholm	Female	1 (2)
Östersund	Male	0.5 (2)
Östersund	Female	1 (3)

Table 3.9: Binomial GLM analysis of impact of location and sex of ladybird origin on onward transmission.

	df	Deviance	AIC	LRT	*P* (>Chi)
None		0.0000	7.3863		
Location	1	2.6341	8.0204	2.6341	0.1046
Sex	1	2.2314	7.6177	2.2314	0.1352

3.3.2 Phenology of the host: sexual contact between generations and general observations on timing of reproduction

3.3.2.1 Temporal sampling in Stockholm in 2011

Overwintered and new generation adult ladybirds in Stockholm in 2011 co-occurred for a period of more than a month (Figure 3.7, City centre locations). Mite infections on the new cohort ladybirds were first observed about three weeks after the start of emergence of the cohort in populations, compatible with onset of reproductive activity (ladybird maturation takes 10 days under optimal food conditions). Mating activity of new cohort ladybirds was directly observed, and commenced at week 6, the point mite infections were first observed.

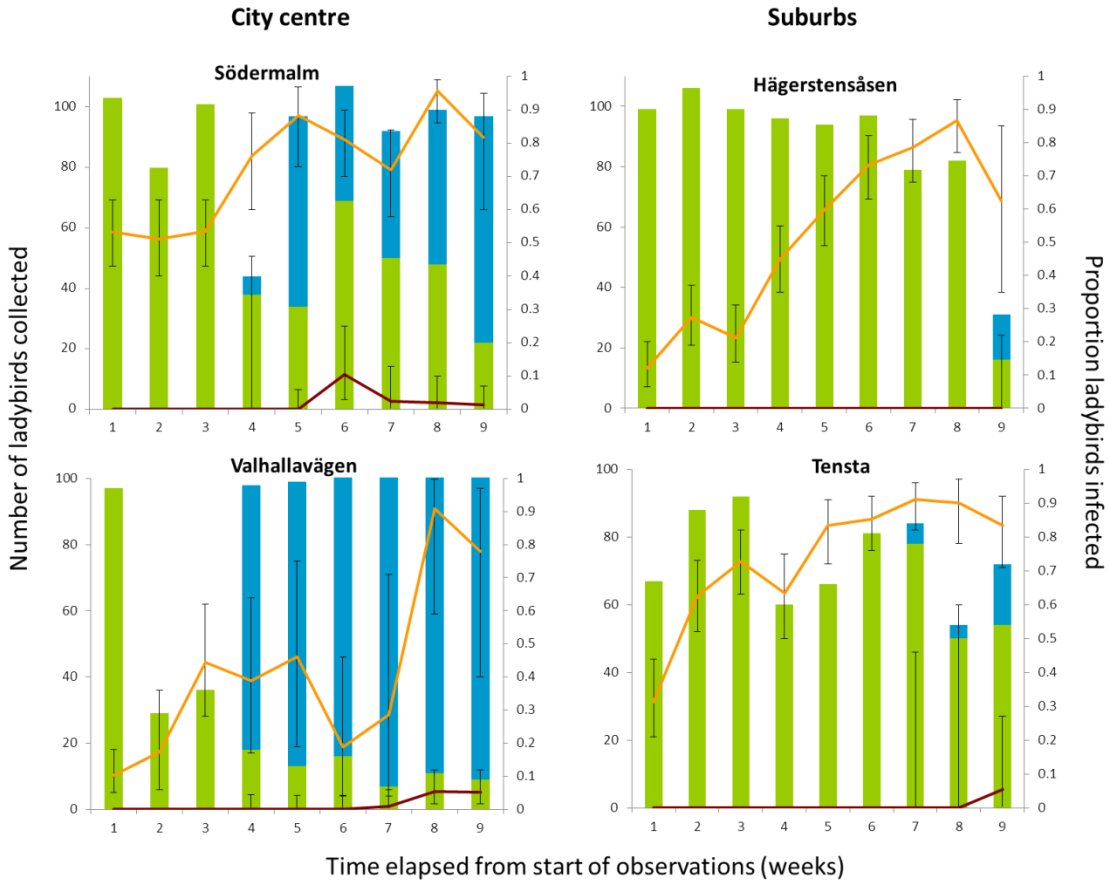


Figure 3.7: Phenology of old and new cohort ladybirds at four locations in Stockholm, Sweden, in Spring/Summer 2011. Bars represent numbers of overwintered (green bars) and new cohort (blue bars) adult beetles. Lines represent prevalence of *C. hippodamiae* in old (orange line) and new cohort (red line) beetles, with binomial confidence interval error bars. X-axis is time elapsed from start of observations (21st May).

Notwithstanding the presence of overlap of cohorts in all locations within Stockholm in 2011, there was obvious spatial and temporal heterogeneity in the pattern of overlap. The reproduction time commenced early in the City centre locations, and new generation ladybirds were evident by the mid of June. In contrast, collections from the suburban locations in this year showed delayed recruitment, with no new generation beetles observed until the first week of July. It is a possibility that the small number observed were immigrants from other locations.

3.3.2.2 Field observations on ladybird populations in August 2012

Observations in 2012 made across Sweden focussed on the state of the populations in August, by which time old cohort ladybirds were expected to be rare or absent. Ladybirds collected in early August 2012 from the southern areas where the mite was naturally present (Gävle and Stockholm) comprised both new and old generation adults, with mite infection established on the both overwintered and new cohort. Between 4% and 16% of new cohort female ladybirds in these locations were fertile, indicating sexual contact had commenced. In these populations, the emergence of the new generation was nearly complete, with very few larvae/pupae remaining on trees. In contrast, ladybird adults collected from areas where mite was not present (Östersund and Ljusdal north of 61˚N, and Nässjö in the south) were solely of the new generation, with no overwintered ladybirds still alive (Table 3.6). The emergence of the new cohort was ongoing, as ascertained from presence of $3^{rd}/4^{th}$ instar larvae and pupae on the trees. None of the females collected in the two northern populations (Östersund and Ljusdal) were fertile. Restoration of fertility following crossing in the laboratory indicated that females in these populations had not entered reproductive diapauses, which could be evidence that this lack of fertility was associated with lack of previous mating activity. In these populations, all females were virgin and there were no old cohort individuals to mate with. Therefore reproductive contact with the overwintered cohort can be excluded. In contrast to the northern populations, 12% of new generation ladybirds in Nässjö (mite absent) had mated. It is not possible to determine whether mated females in Nässjö had sexual contact with old generation males that had since died out, or had mated with new generation males (Table 3.10 and 3.11).

Table 3.10: Phenological observations taken in 2012 at various places in Sweden.

Place	Latitude Longitude	Altitude	Average temperature	Observation date	Mite present	Other ladybird stages present	# overwintered adult cohort collected	# new adult cohort collected
Östersund	63˚11'N 14˚40'E	312 m (1,024 ft)	-10˚C - 19˚C	08/08	No	No eggs. Some fourth instar larvae. Some pupae.	0	99
Ljusdal	61˚50'N 16˚05'E	145 m (476 ft)	-10˚C - 21˚C	09/08	No	Some eggs, many third and fourth instar larvae. Pupae common.	0	92
Gävle	60˚40'N 17˚10'E	68 m (224 ft)	-7˚C - 21˚C	09/08	Yes	None	1	56
Stockholm	59˚19'N 18˚4'E	52 m (171 ft)	-5˚C - 23˚C	11-12/08	Yes	No eggs. Few third and fourth instar larvae. Some pupae.	112	57
Nässjö	57˚39'N 14˚41'E	375 m (1230 ft)	-7˚C - 21˚C	10/08	No	Some eggs, many third and fourth instar larvae. Pupae common.	0	62

Table 3.11: Fertility of new generation females collected from the field in various parts of Sweden in August 2012.

Place	# female tested	# female laid eggs	# female fertile
Östersund	25	8	0
Ljusdal	25	4	0
Gävle	25	5	1
Stockholm	25	24	4
Nässjö	25	17	3

3.3.3 General phenological information on the timing of ladybird reproduction

Phenological records completed for 2011 and 2013 can be found in the Appendix A3.4 and A3.5. For 2012, in which data is most dense, the timing of oviposition can be directly estimated from presence of eggs on trees, and from working back from observations of pupae on trees, with a guide that development to pupation takes c. 21 days at 20˚C.

These data indicate an association between mite presence and early oviposition by the overwintered cohort, with reproduction in mite free populations delayed compared to those where the mite was present (Table 3.12). This delay is observed in both the northern populations, and in Nässjö, the southern population at altitude. The magnitude of delay is greatest in the most northerly populations.

Table 3.12: Phenological observations taken in 2011 at various places in Sweden.

Population	Mite status	Longitude Latitude	Estimated time of first oviposition by overwintered cohort	Evidence
Vilhelmina	Absent	64˚37'N 16˚39'E	After 03/07	No eggs on 03/07
Östersund	Absent	63˚11'N 14˚40'E	After 04/07	No eggs on 04/07
Ljusdal	Absent	61˚50'N 16˚05'E	Between 17/06 and early July	No eggs on 17/06; pupae present on 28/07
Gävle	Present	60˚40'N 17˚10'E	Between 04/06 and 19/06	No oviposition on 04/06; abundant pupae on 08/07
Stockholm	Present	59˚19'N 18˚4'E	Before 21/05	Abundant eggs observed on 21/05
Nässjö	Absent	57˚39'N 14˚41'E	Between 12/06 and 19/06	No oviposition on 12/06; abundant pupae on 09/07
Malmö	Present	55˚35'N 13˚02'E	Before 01/06	Presence of a few pupae on sample 11/06

3.4 Discussion

The sexually transmitted parasite *C. hippodamiae*, is absent from part of its ladybird host species range. This absence provides an opportunity to understand the factors that may underlie the presence/absence of STIs more generally and the role that host age structure may have in determining STI incidence. In Chapter 2, I first established that patterns of mite presence/absence are stable over time. My observations of mite incidence between 2011 and 2013 are precisely congruent with previous records from 2000-2002 presented by Webberley *et al*. (2006b), with Swedish populations north of 61˚N latitude being uninfected with mites, and those south of this latitude being mite infected, with one exception (Nässjö). Thus I can infer a persistent biological or ecological basis to the distribution of the mite.

The absence of the mite from northern populations was not associated with an incompatibility in the 'within host' host-parasite interaction. Ladybirds from north of 61˚N were competent to acquire mites, develop mite infections, and then transmit larval mites onward. Measures of parasite performance on the host, such as latent period and per contact transmission rates, revealed no effect of host origin on the performance of the mite ectoparasite. Therefore, the factors that could explain mite absence in the northern habitats lie in transmission biology associated with host-host contacts. Presence or absence of a sexually transmitted infection may be driven by changes in average mating rate, with low promiscuity preventing STI spread. However, there was no biological difference in promiscuity between beetles from Stockholm (mite present naturally) and from Nässjö (mites absent). Thus, differences in the desire of male/female ladybirds to mate are not supported as driving STI incidence.

Alternately, Knell and Webberley (2004) suggest lack of sexual contact between cohorts as a 'hard stop' to STI persistence. In its most simple sense, an infection that can only exist on adults, and not in the environment, can only persist if there are always adults present. 'Generation gaps' may be associated with life in temperate environments where winter creates defined cohorts of insects that may not overlap, and may also select for diapause in the egg stage. In addition, defined

cohorts of insects may exist in tropical climates through the process of generation cycles (Knell *et al.*, 1998). My observations provide strong evidence for that phenology can create a 'hard stop' to mite transmission within the two northern populations, with no sexual contact between the generations. Only occasional 'hard' stops are required to maintain a condition where mites are not present where host dispersal is limited.

There was support for the hypothesis that differences in host phenology drive incidence. Observations have indicated that populations without the mite are characterized by cohorts that did not overlap and that the overwintered generation of adult ladybirds had died before a new generation adult ladybirds were reproductively mature. Collections of ladybirds from Nässjö, Östersund and Ljusdal in August 2012 contained only new cohort individuals. The females collected from Östersund and Ljusdal showed no evidence of any previous mating activity. As old generation ladybirds were absent in the collections, we can conclude that young females in these populations were overwintering without sexual contact with the previous cohort. This would create a 'hard stop' in mite transmission that would produce an annual fade out of any infection that had arrived through migration. Notably, both the Stockholm and Gävle populations where the mite was present did contain a mix of overwintered and new cohort individuals, and some new cohort females had mated.

Phenological observations suggest two phenomena contribute to the lack of sexual contact between old and new generation ladybirds in northern populations. First, reproduction commences earlier in the mite-present populations, such that the new generations emerges earlier. Second, old generation ladybirds die off more rapidly in the northern populations despite their later emergence from overwintering. There are no overwintered beetles in Östersund (63˚11'N 14˚40'E) or Ljusdal (61˚50'N 16˚05'E) in the early August samples, and 'edge of range' population, Gävle (60˚40'N 17˚10E), had a single overwintered cohort beetle at this time. Thus, I conclude that there are two forces that separate the overwintered and new cohort in the North. First, a later exit from diapause is evident, such that new generation

ladybirds arrive later in the year. Second, the overwintered ladybirds survive less long into the season, despite emerging later.

Phenological evidence from the 'mite present' populations was also consistent with presence of sexual contact between generations. Temporal data from the Stockholm population in 2011 clearly indicated substantial overlap between cohorts. It is notable that there is, nevertheless, spatial heterogeneity in overlap within Stockholm. The cohort overlap was pronounced in City centre sites and only weakly present in the suburbs. These locations are within 12 km of each other, and indicate that spatial variation in host phenology represents an important buffer against generation gaps. To this data is added information from August 2012 collections. In these, both the Stockholm and Gävle populations contained a mix of overwintered and new cohort individuals during the August 2012 collections, and some new cohort females had mated. Thus, sexual contact between new and old cohort individuals is highly likely to have occurred in this year as well.

The ladybird population from Nässjö (57˚39'N, 14˚41'E), which is located south of Stockholm, presents a more ambiguous case. It is difficult to explain the absence mite in this location. This city is a part of the Highlands of Southern Sweden called Småland and lies at 375 metres (1230 ft) above sea level. Like the northern mite absent populations, the overwintered cohort had died off by August 2012. However, in contrast to these populations, recruitment of the new cohort begins earlier in the year (equivalent to the northernmost mite-present population of Gävle, but delayed compare to Stockholm and Malmö). I did note some fertility of new cohort females in this site, but it was unclear if this was associated with sexual contact with the overwintered cohort (it may have been derived from contact between new generation ladybirds). It is likely that Nässjö is on the margins of being able to retain mite infection, but that transfer between cohorts is impossible in a fraction of years, such that the mite is generally absent.

In summary, the incidence of STIs in *Adalia* is associated with the presence of reproductive continuity between generations (Webberley *et al.,* 2002; Knell and Webberley, 2004; Webberley *et al.,* 2004; Webberley *et al.,* 2006b). More widely, I

would expect the incidence of STIs that are confined to adult host, have lower (or limited) environmental survival for univoltine species towards Polar regions, as the reproductive period becomes more compressed and the time cohorts overlap decreases. It is notable in Europe, for instance, that many species show an obligate diapause requirement in the north, such that they will not engage in sexual activity until emergence from overwintering. I would predict that for ladybird species such as *Coccinella septempunctata*, (Phoofolo and Obrycki, 2000; Hodek *et al.*, 2012), where there is genetic requirement for overwintering in northern regions, the incidence of the sexually transmitted mite *Coccipolipus macfarlenei* would correspond to the geographical region where there is no diapause requirement.

Chapter 4: No evidence that presence of sexually transmitted infection selects for reduced mating rate in the two-spot ladybird, *Adalia bipunctata*

1. Sexually transmitted infections (STIs) are common in animals and plants, and frequently impair individual fertility. Theory predicts that natural selection will favour behaviours that reduce the chance of acquiring a STI.
2. I investigated whether an STI, *Coccipolipus hippodamiae* has selected for a reduced rate of remating by its host *Adalia bipunctata* as a mechanism to avoid exposure.
3. I first demonstrated that rejection of mating by females did indeed reduce the chance of acquiring the mite.
4. I then examined whether rejection rate and mating rate differed between ladybirds from mite-present and mite-absent populations when tested in a common environment.
5. No differences in rejection intensity or remating propensity were observed between the two populations.
6. I also observed that the level of promiscuity of ladybirds from the mite-absent population of Nässjö was equivalent to that of ladybirds from Stockholm, where the mite is present.
7. I therefore conclude there is no evidence that STIs have driven the evolution of mating systems in this species.

A modified version of this chapter has been published at *PeerJ*:
Jones, S., Pastok, D. & Hurst, G. D. D. (2015) "No evidence that presence of sexually transmitted infection selects for reduced mating rate in the two spot ladybird, *Adalia bipunctata*." PeerJ 3:e1148 https://dx.doi.org/10.7717/peerj.1148.

4.1 Introduction

Many animals carry pathogens and parasites which cause sexually transmitted infections (STI) and they are primarily transmitted during copulation. STIs have been recorded in 73 species of STI infect 182 different insect species. Most commonly, the infections are multicellular ectoparasites: mites, nematodes and fungi that live on the outside of the individual and transmit between partners during copulation (Knell and Webberley, 2004).

Sexually transmitted infections commonly have a relatively small negative effect on host mortality. However there is nevertheless a significant severe effect on its host fitness because they either reduce host fecundity or sterilise their host (Hurst *et al.*, 1995; Lockhart *et al.*, 1996; Apari *et al.*, 2014). Natural selection may therefore favour host traits that reduce the risk of infection. Selection is expected to be particularly strong on female hosts, because:

a) Each additional mating adds little to fitness compared to males (the Bateman gradient)

b) Impacts on female fitness are higher than on male fitness, as STIs commonly sterilize female hosts, with less impact on male hosts.

In ladybirds, for instance, there is strong last male sperm precedence, such that frequent mating by a male will result in more progeny sired. Laboratory assays show female fitness, in contrast, is only weakly affected by remating, with a single mating providing enough sperm for 7-10 days oviposition activity (Haddrill *et al.*, 2007). The impact of the STI is also greater in the female than in the male, with females sterilized by the STI (Hurst *et al.*, 1995), a phenotype not observed in male hosts.

There are three possible behavioural routes to reducing the chance of a female acquiring an STI. First, a female can mate less frequently. Theory predicts that STI presence should select for an increase in female refusal to mate when courted (Boots and Knell, 2002; Kokko *et al.*, 2002). Second, a female could specifically reject matings with males carrying an STI. Rejection of mating then represents a

'contagion avoidance' mechanism (Borgia, 1986; Able, 1996). Third, a female could employ post-copulatory behaviours (such as grooming, urination) to reduce the chance of acquiring the STI following mating to an infected partner.

Previous empirical research has focussed on the latter pair of mechanism: rejection of infected partners, and post-copulatory grooming. Rejection of infected partners has been tested in a variety of systems (including in ladybirds) (Abbot and Dill, 2001; Nunn, 2003; Webberley *et al.*, 2002). It is possible that the absence of choice based on STI presence is a product of STIs being selected to be cryptic (Knell, 1999), a process supported by observations on syphilis in European populations (Knell, 2004). Post-copulatory grooming processes have been examined in some mammals, and some behavioural data (e.g. male masturbation following copulation) suggested as adaptations to prevent STI transmission (Hart *et al.*, 1988; Waterman, 2010).

In contrast, there has been little empirical study on the impact of STIs on the evolution of mating rate. The expectation is that species with an STI will be selected for lower mating rate than species without an STI, and that, where populations of a species vary in STI presence/absence, those with the STI are selected for reduced mating rate. The geographical variation in STI presence in *Adalia* (Chapter 2), accounted for by variation in host phenology (Chapter 3) provides an excellent basis for such a comparison. *Adalia* females can reject matings through running away from males, through raising their abdomen within their elytra (preventing male access), and through rolling over to dislodge a male (indeed, also, falling off a leaf to land on the male). In this chapter, I first examine whether rejection behaviours prevent mite transmission, a necessary requirement for rejection to evolve as a means of preventing mite acquisition. Following this, I used laboratory experiments to test the hypothesis that rejection behaviours (and lower mating rate) are more commonly seen where the beetles derive from populations in which the STI is present, compared to ones where it is not.

4.2 Material and methods

4.2.1 Does rejection of mating by a female prevent transmission of C. hippodamiae infection?

In the first place, I examined whether recently collected infectious beetles of both sexes transferred mites to their partner during rejected matings compared to successful matings. Female and male ladybirds were collected from Stockholm in June/July 2011 and returned to the laboratory. Mostly they belonged to overwintered generations with few newly emerged individuals. Collected ladybirds were classified as being uninfected, latent infected or infectious on the basis of absence of mites, presence of mites without infectious larval mites, and presence of larval mites ready to transmit respectively. Pairs comprising a single infectious male with a focal uninfected female, and a single infectious female with a focal uninfected male were established in clean 90 mm diameter Petri dishes in the laboratory, and behaviour observed for 30 minutes. Behaviour was scored as 'no interaction', 'rejected mating', and 'successful mating'. Pairs that mated were allowed to mate to completion before separation of the focal partner to a new Petri dish. The focal individual was then examined 24 hour later for the presence of larval mites (Figure 4.1).

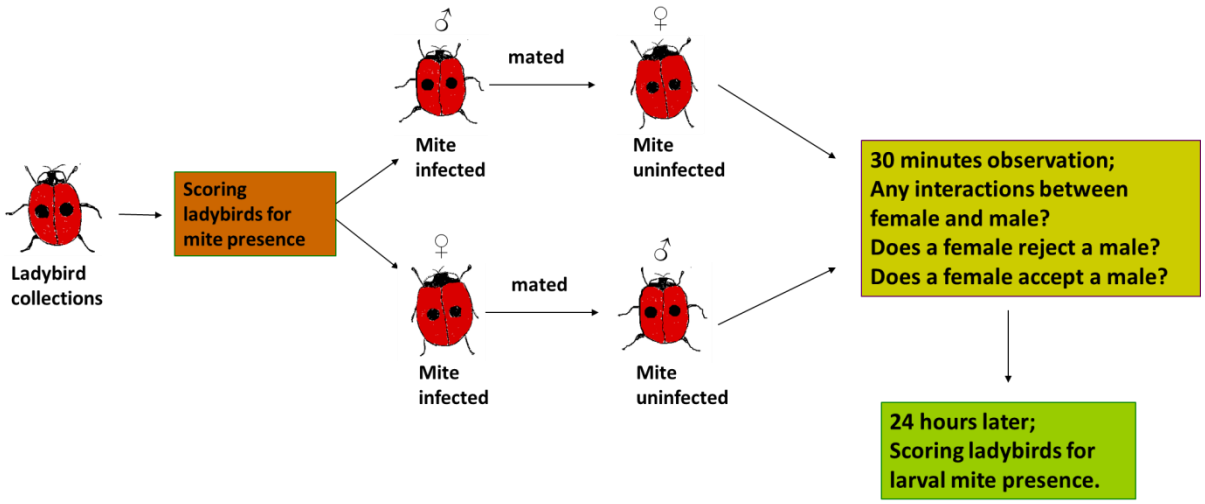


Figure 4.1: Experimental design: Testing whether rejection behaviours prevent mite transmission (wild ladybirds).

Statistical analysis

The importance of focal host sex and mating/rejection behaviours on mite transfer was analysed with a binomial GLM. The first model included an interaction term between focal host sex and mating/rejection behaviour. However this term was not significant (i.e. the effects of mating/rejection do not depend on focal host sex) and was removed from the further analysis. The effect of the factor focal host sex and mating/rejection were examined.

4.2.2 Do ladybird females from populations that carry the STI show lower mating rates and a greater likelihood of rejecting mating? Comparison of promiscuity of A. bipunctata from Nässjö and Stockholm

This experiment was part of the undergraduate project conducted by Sophie Jones and me, and run by Sophie Jones. *Adalia bipunctata* were collected in Sweden from two locations approximately 300 km apart during August 2012: Nässjö (57.7˚N, 14.7˚E) and Stockholm (59.3˚N, 18.1˚E). The Nässjö population is free from mite infection (Webberley *et al.*, 2006b) whereas in Stockholm there has been recorded an annual epidemic of the mite infection, leading to nearly all overwintered ladybirds becoming infected by June/July (Ryder *et al.*, 2013; Ryder *et al.*, 2014). Females from these populations were allowed to mate with sympatric males. Eggs laid from these females were collected, and larvae reared on a diet of pea aphids. The rearing was conducted for both populations to standardize environment. The hatched adult ladybirds were first scored for sex and then maintained in single sex 90 mm diameter Petri dishes at 20˚C for 30 days before the experiment to allow them to mature. In the natural populations of two-spot ladybirds most individuals become reproductively mature a week after they hatch (Majerus, 1994). However here in this experiment I left ladybirds for 30 days to be sure that ladybirds from both Stockholm and Nässjö populations were mature enough and there were no differences between them in their willingness to mate. Ladybirds were provided with a diet of pea aphids daily to ensure they were in reproductive condition by the time the experimental observations commenced. All behavioural observations

occurred in the absence of mites to avoid any direct impact of mites on the mating behaviour of their host (Webberley *et al.*, 2002).

To test the propensity to remate, 'populations' of five females and five males were created. Individuals were unrelated and all were virgin. Three days before the experiment within each population, males and females were mixed randomly and were allowed to mate. This was intended to reduce artefactual behaviour resulting from single sex confinement.

Subsequently, each female from each population (Stockholm and Nässjö) was paired with a male for 30 minutes at the same time each day for a five day period. Each pair was placed in a clean Petri dish and was kept at 21˚C for the duration of the observation. The presence of the following behaviours was observed:

1. The number of interactions between female and male
2. The presence and duration of observed rejection behaviours
3. Whether the interactions resulted in mating.

Females were offered a different male within their population every day (see Table 4.1 for block design).

From these measures, the propensity of females to remate, the likelihood of a female rejecting mating, and the probability of successful mating occurring, were calculated. This block design was replicated four times for each population resulting in 20 females being tested for each population. For simplicity, all blocks were treated together in the statistical analysis. All contingency tests were carried out in Minitab 16 Statistical Software (Minitab Inc.). All error bars for proportionate data represent binomial sampling intervals calculated using the Clopper–Pearson method (1934) (http://www.danielsoper.com/statcalc3/calc.aspx?id=85).

Table 4.1: Five day experimental block design of sympatric matings between Stockholm (SF=Stockholm Female, SM=Stockholm Male) and Nässjö (NF= Nässjö Female, NM= Nässjö Male) individuals. Numbers in the matrix indicate day of mating.

	SF1	SF2	SF3	SF4	SF5
SM1	5	4	3	2	1
SM2	1	5	4	3	2
SM3	2	1	5	4	3
SM4	3	2	1	5	4
SM5	4	3	2	1	5

	NF1	NF2	NF3	NF4	NF5
NM1	5	4	3	2	1
NM2	1	5	4	3	2
NM3	2	1	5	4	3
NM4	3	2	1	5	4
NM5	4	3	2	1	5

4.3 Results

4.3.1 Does rejection of mating by a female prevent transmission of C. hippodamiae infection?

Transmission rates from wild-caught infectious male and female individuals to uninfected partners with which they mated were high, with only one of 26 females not acquiring infection during mating with an infectious male partner, and one of 35 males not acquiring infection from an infectious female partner. In contrast, transmission was rare when mating was rejected, with one of seven females acquiring an infection following rejection of the infectious male, and one of three males acquiring infection having been rejected by an infectious female. Statistical analysis revealed there was no effect of donor sex on the mite transmission probability (GLM factor 'host sex', P=0.288), however there was a significant effect of the factor 'rejected/mated' behaviours on the mite transmission (GLM factor 'mated/rejected', P<0.0001) (Figure 4.2 and Table 4.2).

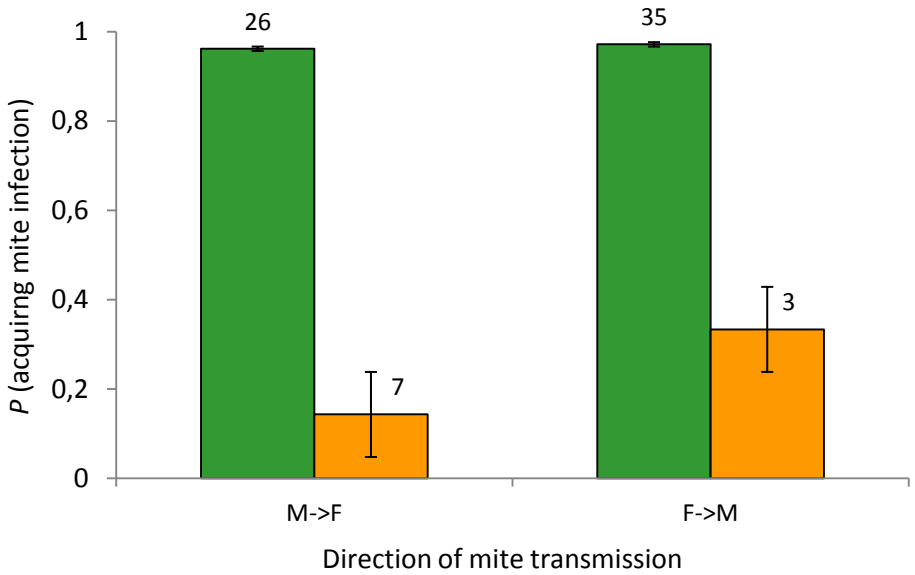


Figure 4.2: Mite transmission rate observed from wild infectious ladybirds to uninfected partners. Green colour bars ● represents mite transmission rate on ladybirds which accepted their partner and mated. Orange colour bars ● represents proportion of rejecting females that acquired mites. Directions of mite transmission

were from male (M) to female (F) and from female (F) to male (M). Number above the bars represents the sample size.

Table 4.2: GLM model for the response variable presence/absence of mite transmission with donor sex (female/male) and accepted/rejected behaviours as factors.

Factor	Df	Deviance	*P*
Female/Male donor sex	1	1.1302	0.288
Accepted/Rejected behaviours	1	25.1896	<0.0001

4.3.2 Comparison of promiscuity of A. bipunctata from Nässjö and Stockholm

4.3.2.1 Is there an association between location and mating rate?

Mating was observed to be more common on day 1 than on other days in experiments involving both Stockholm and Nässjö (Figure 4.3). Combining over blocks, and between locations, mating was heterogeneous over the experiment (χ^2=16.042, df=4, *P*=0.003). This diversity was associated with high mating rates on day 1 (after 3 days without mating activity); when day 1 was excluded from analysis, mating rates was homogenous over days 2-5 (χ^2=0.276, df=3, *P*=0.964). Thus, in further analysis, day 1 mating was excluded, as the high mating rate on this day was likely to be associated with experimentally induced lack of mating opportunity in previous days. Analysis across days 2-5 revealed that there was no evidence of an association between location and remating rate (χ^2=0.627, df=1, *P*=0.428).

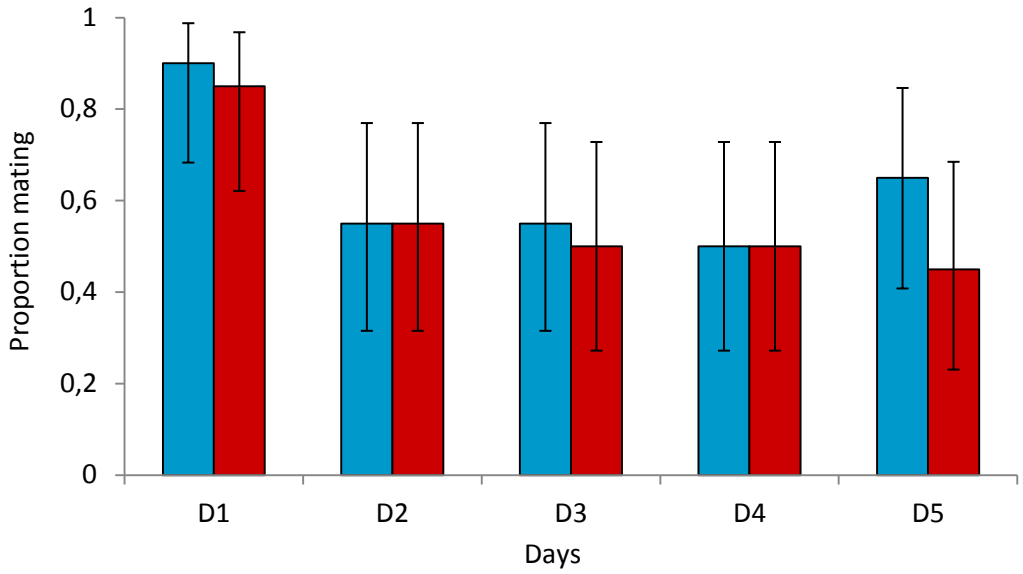


Figure 4.3: Proportion of pairs that mated each day during 30 minute period from Stockholm (blue bars ●; STI naturally present, though absent in the laboratory) and Nässjö (red bars ●; STI absent). *N*=20 for all days.

4.3.2.2 Is there an association between location and rejection rate?

Rejection behaviour was categorised into different intensity levels; no rejection observed; mild rejection (<1 minute); moderate rejection (1-5 minutes) and intense rejection (>5 minutes). Most females exhibited some rejection behaviour, and this was prolonged in over half of cases in both populations. There was no evidence that females from the two populations differed in the intensity of rejection behaviour following a males attempt to mate (χ^2=4.878, df=3, *P*=0.181) (Figure 4.4).

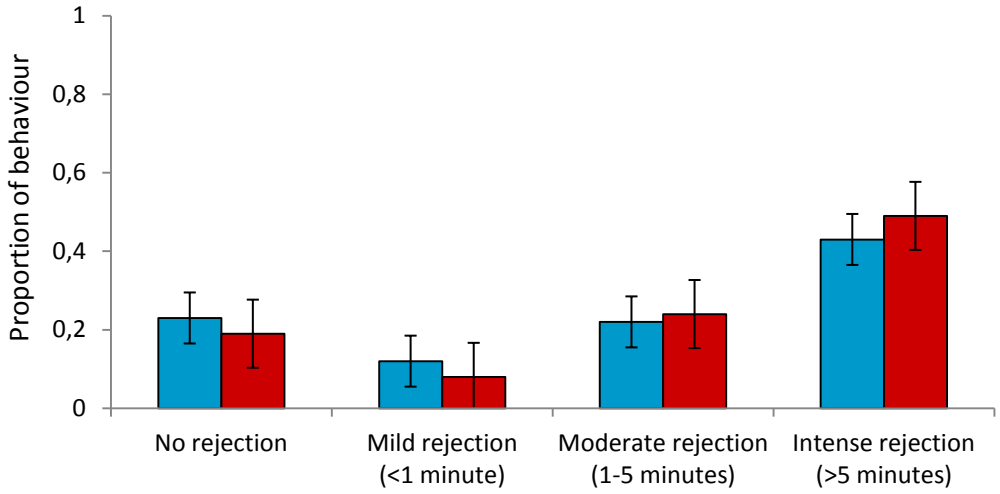


Figure 4.4: Proportion of different intensities of rejection behaviour (No rejection, mild rejection (<1minute), moderate rejection (1-5minutes), intense rejection (>5minutes)) observed from Stockholm (blue bars ●) (STI naturally present, though absent in the laboratory) and Nässjö (red bars ●) (STI absent) females during 30 minute period experiments over days 2-5. *N*=64 for both populations.

4.3.2.3 Would mating rate differ if only first interaction between male and female counts and the intense rejection is counted as a failed mating?

The confined environment of the Petri dish allows males repeated interactions with the female that are unlikely to occur in the field. Further, rejection behaviour is likely to be less efficient in the laboratory environment, as some behaviours (e.g. falling off leaves, rolling over) are either not possible or less efficient. Data on mating rate was reanalysed to create a more environmentally relevant measure of mating rate ('environmental' mating rate) that examined the result of the first interaction only, and discounted mating if this took more than five minutes to achieve.

The 'environmental' mating rate for Stockholm and Nässjö was half that of the overall mating rate (Figure 4.5). Analysis indicates there was no evidence of association between location and 'environmental' mating rate (χ^2=0.295, df=1, *P*=0.587).

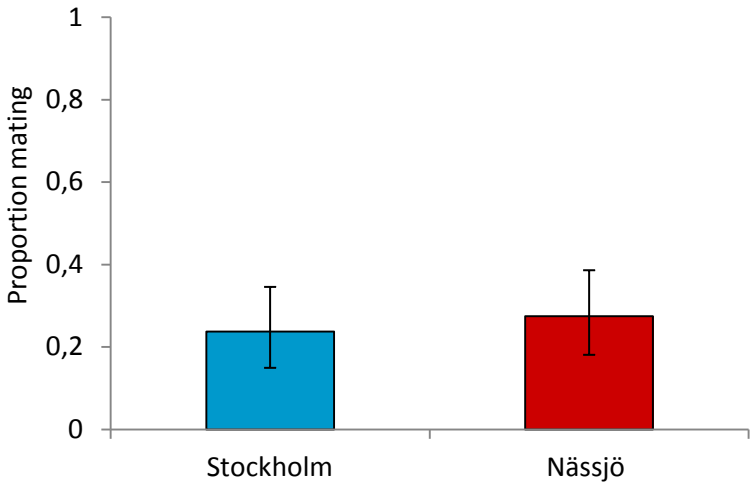


Figure 4.5: Proportion of ladybird pairs that mated during 30 minute period from Stockholm (●) (STI naturally present, but here absent in the laboratory) and Nässjö (●) (STI absent) over days 2-5. Data were reanalysed and only pairs that mated on the first interaction and exhibited rejection behaviour for less than five minutes were considered successful matings. *N*=80 for both populations.

4.3 Discussion

Sexually transmitted infections (STIs) are very common in the animal kingdom and they frequently have deleterious impacts on their host (Lockhart *et al.*, 1996,; Knell and Webberley, 2004). The presence of STIs has been widely conjectured to select on the host mating system and mating behaviour, through avoidance of mating, discrimination against infected partners, and through post-copulatory grooming. In this chapter, I investigated the hypothesis that females from populations where mites were naturally present used rejection as a means to prevent infection. I first used laboratory experiments to investigate whether rejection behaviour was a barrier to mite transmission, and then assessed whether this behaviour was more commonly observed in populations where the mite was present.

Rejection behaviour in *Adalia* was efficient at preventing mite transmission. In experiments using field-collected beetles, over 95% of individuals that mated with an infected partner acquired an infection, compared to two of ten individuals that were approached but rejected mating. The two individuals that did acquire mites carried very few mite larvae, and low intensity infections such as this are much less likely to be retained (Chapter 6). Thus, rejection is an efficient (though not completely efficient) means of preventing STI exposure/STI induced sterility. Female *A. bipunctata* can show quite extreme rejection behaviour, to the extent that they will jump off foliage with a mounting male in an attempt to dislodge him. Rejection occurs particularly commonly when females are poorly fed (Perry *et al.,* 2009), and it is known that mating in the absence of food can be particularly costly (Perry and Tse, 2013). This data suggest that rejection behaviours may additionally function to reduce STI acquisition rates under conditions where reproduction is not possible, and also that the refractoriness induced in females immediately following mating may have benefits in reducing her STI exposure, notwithstanding any benefits to the male in protecting paternity.

In contrast, there was no evidence that rejection behaviour was more commonly observed in interactions between beetles from Stockholm (mite present) than between those from Nässjö (mite absent). When individuals are provided with one

mating opportunity per day, rejection behaviour is commonly observed (122 of 128 interactions where the male approached the female and attempted to mate). The frequency and intensity of rejection behaviour did not differ, and likewise the likelihood of the pair mating was not different between beetles from Stockholm than between those from Nässjö. Therefore, the hypothesis that female mating behaviour has been selected to avoid the mite infection was not supported by my observations and data, despite ladybird rejection behaviours being partly effective at preventing the STI transmission.

Failure to find a difference in remating rate between populations from Stockholm and Nässjö could have three sources. Firstly, there may be no difference. Secondly, a difference may be present but its effect is small. However, it is notable my observations revealed a relatively higher mating rate for ladybirds from Stockholm, where the mite is naturally present, than from Nässjö. Third, the experiment may not be suitable to detect a difference in mating rate; for instance, ladybirds were not under natural environmental conditions which may limit the potential to infer outcomes in wild populations. Even though the experimental design replicated those natural conditions which are required for mating to happen (e.g. temperature, the length of day and source of food), it is possible that the unnaturally frequent repeated sexual interactions, or other conditions that lead to ineffective behaviour, could disrupt normal ladybird mating behaviours.

On the other hand if we consider only the first mating attempt between ladybird female and male, I can indeed conclude that rejection behaviours did not differ between those two populations. These results could suggest that the STI has not led to the evolution of an altered mating system between populations where the mite is present and where the mite is absent.

There may be a few explanations for why the mating system hasn't evolved in response to the presence of the STI in the Stockholm populations. One possibility is that a high mating rate is required for female fertility, such that females who refuse to mate incur a cost. However, *Adalia bipunctata* females mated singly have equivalent fertility, measured over 20 days, to females mated every two days

(Haddrill *et al.*, 2007). Thus, there is plenty of scope for a female's risk of mite-induced infertility to be reduced before sperm-depletion associated infertility is observed. A second possibility is that local adaptation is not possible in this species. However, the presence of variation in the frequency of colour pattern variants in this species on equivalent spatial scales (Brakefield, 1984) make it unlikely that gene flow sufficient to impede local adaptation. A third hypothesis is that selection to prevent the STI does operate in the way expected, but there are other factors differing between populations that influence mating rate evolution. It is possible that there is a counterbalancing selective force working in opposition to the effect of the STI. The source of such selection is not obvious (the two populations use similar habitat and have a similar sex ratio), but such a hypothesis cannot be ruled out. Finally, the prediction that STIs select for lower mating rate applies to female hosts, in which there are smaller benefits to each additional mating, and in this species, higher costs of infection (sterility). Selection on males is not expected to act in the same way, as each mating provides significant fitness benefits, and the STI is only weakly costly to the male host (Ryder *et al.*, 2007). If mating rate is determined by males, then the STI is less likely to drive mating system evolution.

In summary, the experiment demonstrated that rejection behaviours was partially efficient at preventing STI transmission, but did not occur more commonly in ladybirds derived from populations where the STI was common. This study, combined with previous analysis indicating that STI infected ladybirds were not disadvantaged in acquiring mates (Webberley *et al.*, 2002), produces no support for the hypothesis that mating behaviour evolves in response to the presence of a sterilizing STI. An intriguing possibility is that STIs are most commonly observed in species in which evolution to resist STI transmission is inhibited.

Chapter 5: *Spiroplasma* do not alter STI epidemiology through protective or phenological effects

1. Symbionts are known to affect their host's susceptibility to natural enemies, for instance conferring protection on their host. Other symbionts alter host demography in such a way that they may affect onward transmission of infection, for instance through changing lifespan.

2. In this study I tested whether a *Spiroplasma* symbiont can affect the susceptibility of its host, the two-spot ladybird (*Adalia bipunctata*) to infection with the sexually transmitted mite, *Coccipolipus hippodamiae*, and whether *Spiroplasma* induces changes in ladybird longevity that may impact upon the chance of inter-cohort transmission of the mite.

3. One indicator of protection is the presence of an association between parasite presence and lack of a symbiont. Field data collected in Stockholm in one year indicated the presence of an association between mite and *Spiroplasma* presence, where *Spiroplasma*-infected ladybirds were more likely to carry mites. However this association was not observed in any other Stockholm collection seasons or in other Swedish cities. Therefore the significance of the association is uncertain.

4. Laboratory study of mite acquisition and transmission, and impacts on the host mortality and fertility, provide no evidence for a direct *Spiroplasma* influence on ladybird susceptibility to mite infection.

5. Laboratory study of the longevity of field-collected overwintered ladybirds provided evidence for a complex effect of *Spiroplasma* on longevity. Females lacking symbionts died more rapidly than females with symbionts, but only in the absence of the mite.

6. I conclude that there may be direct effect of *Spiroplasma* on the two-spot ladybird through increases in longevity, which may affect mite persistence through enabling transmission between cohorts of the host.

This chapter has been published in part as:
Ryder, Hoare, Pastok *et al.*, "Disease epidemiology in arthropods is altered by the presence of nonprotective symbionts". *American Naturalist*, 183: E89-104 (2014).

5.1 Introduction

Parasitism is a very common interaction between two organisms where one (the parasite) lives on and exploits the second (the host). The diseases produced by parasites create strong natural selection on the host for resistance or tolerance of the parasite. Recently, it has emerged that heritable symbionts form an important component of defence against natural enemies (Haine, 2008). This type of interaction is commonly known as symbiont-mediated protection (or microbial-mediated protection) and is widespread in many organisms from insects, to humans (Kamada *et al.,* 2013) and plants (Mendes *et al.,* 2011).

According to Haine (2008) protective symbioses can be separated into three categories relating to the type of organisms against which the protection is directed. There are groups of symbionts which defend their host a) from pathogens; b) from parasites and c) from predators. The first group is characterized by symbionts which are able to protect their host against microorganisms that have a pathogenic effect. For instance Ferrari *et al.* (2004) and Scarborough *et al.* (2005) observed host aphids become resistant to fungal pathogen *Pandora neoaphidis* in the presence of the facultative symbiont *Regiella insecticola*. In another example, *Wolbachia* is protective for *Drosophila melanogaster* against attack of dsRNA *Drosophila C Virus* (DCV) (Hedges *et al.,* 2008). This effect is not restricted to the body cavity. Study on bumblebees (*Bombus terrestris*) has revealed that its gut microbiota community provides defence against the trypanosomatid gut parasite *Crithidia bombi* (Koch and Schmid-Hempel, 2011).

In the second group are symbionts that guard their host against parasitic invertebrates. The facultative bacterial symbionts, *Serratia symbiotica* and *Hamiltonella defensa,* protect the pea aphid *Acyrthosiphon pisum* against the parasitic wasps *Aphidius ervi* and *Aphidius eadyi,* by increasing mortality of parasitoid wasp larvae (Oliver *et al.,* 2003; Ferrari *et al.,* 2004; Oliver *et al.*, 2005). *Spiroplasma* can protect fly hosts, for instance *Drosophila neotestacea* against attack by nematode worm parasites such as *Howardula* (Jaenike *et al.,* 2010a) or *Drosophila hydei* against a parasitic wasp, *Leptopilina heterotoma* (Xie *et al.,* 2010).

On the other hand there are also some symbionts that increase susceptibility to infection and parasites. Certain strains of *Wolbachia* for instance can make *Spodoptera exempta* army worms more susceptible to *Nucleopolyhedrosis* SpexNPV virus attack (Graham *et al.,* 2012). In another study Fytrou *et al.* (2006) has shown that *Wolbachia* does makes its host *Drosophila simulans,* more susceptible following attack by the parasitic wasp *Leptopilina heterotoma*.

To the third group belong symbionts which produce toxic substances which can defend hosts from predators. For instance the bacterial symbiont *Endobugula sertula* releases a polyketide which protects its bryozoan and isopodan hosts from predation by fish (Haine, 2008). Defence of rove beetle larvae, *Paederus sabaeus*, against predators likewise use a polyketide pederin produced by an endosymbiotic bacterium *Pseudomonas aeruginosa* (Kellner, 2002).

The impact of symbionts on the epidemiology of other infections in the host extends beyond direct effects on host susceptibility. They may additionally be mediated by changes in demography or phenology. Relevant here is the analysis of Elgnady *et al.* (2013) on the impact of *Spiroplasma* on the longevity of *Adalia bipunctata*. In this study, 50% of adult females not infected with *Spiroplasma* died by around 60 days, whereas 50% of *Spiroplasma*-infected ladybirds died by 170 days. The changes in longevity, if observed in natural populations, would alter host phenology, leading to overwintered ladybirds in *Spiroplasma*-infected populations living longer into the season, and establishing sexual contact with the newly emerged generation. A longevity effect of this kind would have implications for mite epidemiology, and indeed incidence.

In this chapter my aim was to test a) whether presence of *Spiroplasma* has a direct effect on mite infection through altering individual susceptibility to mite infection; b) whether *Spiroplasma* increases longevity of ladybirds taken from the field following overwintering in such a way that it could increase the degree of contact between overwintered and newly emerged cohorts.

5.2 Material and methods

5.2.1 Does the symbiont stop the host from acquiring and transmitting the mite?

Spiroplasma influences on mite acquisition and transmission were assessed in two ways. First, the pattern of infection of mite/*Spiroplasma* in natural populations was assessed, with the hypothesis that *Spiroplasma*-mediated protection would be evidenced by lower mite prevalence on *Spiroplasma*-infected host individuals. Second, laboratory experiments were used to directly test for an influence of *Spiroplasma* on mite acquisition or the development/transmission of infection, or the sterility phenotype.

5.2.1.1 Are Spiroplasma-infected ladybirds less likely to carry mites in the wild?

Two-spot ladybirds were collected during June and July 2011 in Sweden from Gävle, Stockholm and Malmö mainly from lime trees (*Tilia sp.*) using a beating tray. Ladybirds were transferred to Eppendorf tubes. In the laboratory ladybirds were scored for sex using morphological characteristics described by Randall *et al.* (1992) and mite presence by examining elytra under a binocular microscope. *Spiroplasma* prevalence in females was assessed using molecular assays as previously described in Chapter 2. Data was analysed to see if there is an association between presence of *Spiroplasma* symbiont and mite infection in females in the Swedish ladybird populations (Figure 5.1).

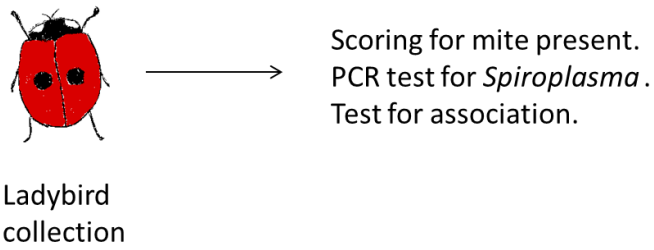


Figure 5.1: Testing if there is any association between *Spiroplasma* and mite presence on wild ladybirds.

5.2.1.2 Does Spiroplasma produce resistance to mite infection in ladybirds in the laboratory environment?

Ladybirds were collected in Stockholm (Sweden) between June and July 2011. They were transported to the laboratory in Eppendorf tubes and scored for sex and mite presence. I examined if *Spiroplasma* alters the ability of its host females to acquire and retain mite infection, and whether the infertility impact of mite infection was affected by *Spiroplasma* presence. Therefore I prepared an experiment in two blocks. In the first block, infectious ladybirds of both sexes (donor) collected in Sweden from Stockholm and Gävle 2011, were paired with uninfected ladybirds of the opposite sex (recipient) and were allowed to mate. In the second block mite uninfected ladybird females only (recipient), which were collected in Stockholm 2011, were also paired to an infectious male partner (donor). This test was conducted to produce greater power to analyse any effect of *Spiroplasma* on the persistence and acquisition.

To this end, those pairs were left for an hour in single Petri dishes. The presence of mating was recorded. If some ladybirds were still mating after an hour, they were allowed to finish copulation. After mating ladybirds were separated to new Petri dishes to avoid any post-mating mite transmission. Recipient ladybirds were transferred to the incubator at 22˚C on a 20L:4D light cycle (20 hours of light and 4 hours of darkness when the temperature decreased to 10˚C) and they were fed aphids daily. The next day recipient ladybirds from two blocks were scored for mite presence and later the progress of mite development on those ladybirds was monitored. Mite infection was described as successful when larval mites were produced on these infected ladybirds. Ladybird females from block two were also checked for intensity of mite infection (how many adult mites were produced) 30 days after the beginning of the infection (Figure 5.2).

During that experiment the following measurements were taken:

a) Transmission efficiency during mating (the proportion of recipients which acquired mite within 24 hours after mating);

b) Recovery from infection (the proportion of recipients which became mite-infected but later lost infection during the experiment);

c) Mortality (the proportion of ladybirds which acquired mites but died before becoming infectious)

d) Latent period of infection. On the seventh and fourteenth day individuals were checked for the progression of the mite infection. After day 19, the ladybirds were checked daily for the presence of hatching larval mites that indicate infectivity. Latent period was calculated as the time between initial infection of the host and the emergence of the first generation of female mite larvae underneath the ladybird elytra.

Ladybird females from each block were tested for *Spiroplasma* presence *post-hoc* using PCR assays previously described in Chapter 2.

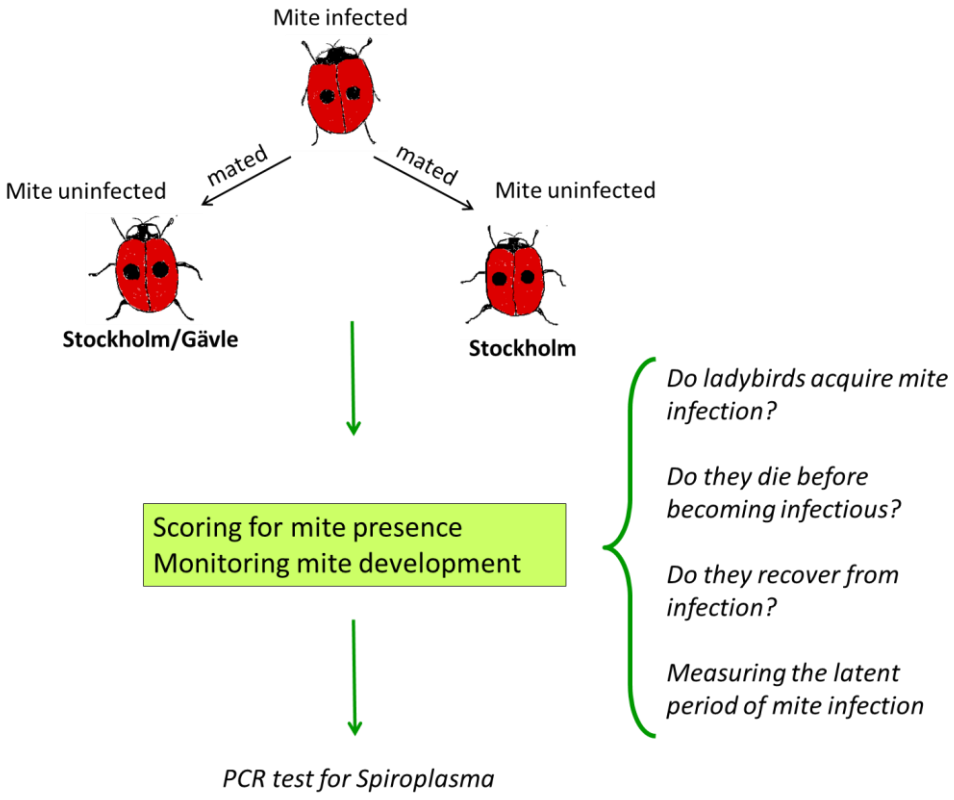


Figure 5.2: Testing if *Spiroplasma* bacteria make ladybirds resistant to the mite infection in the laboratory environment.

5.2.2 Does Spiroplasma protect the host against mite-induced infertility?

5.2.2.1 Are Spiroplasma-infected females in the wild fertile despite mite infection?

Adalia bipunctata were collected in Stockholm (Sweden) between June and July 2011. They were then transported to the laboratory in Eppendorf tubes and were scored for sex and mite presence under a binocular microscope. Mite-infected females and females free from mites (control) were separated to single Petri dishes and were allowed to lay eggs. When a female laid a clutch of eggs, she was transferred to a new Petri dish. Uninfected males were provided to minimize infertility from lack of sperm. Three clutches from each female were collected. The number of eggs laid by females was recorded and these eggs were kept in the incubator at 22˚C. All these eggs were tested for fertility. If any eggs hatched into larvae, the female was scored as fertile. If eggs fail to hatch and became shrivelled, the female was scored as infertile.

When three egg clutches were collected, the *Spiroplasma* infection status of the female was ascertained by PCR assay, as previously described in Chapter 2. The fertility impact of the mite, *Spiroplasma* and the mite-*Spiroplasma* interaction was then tested (Figure 5.3).

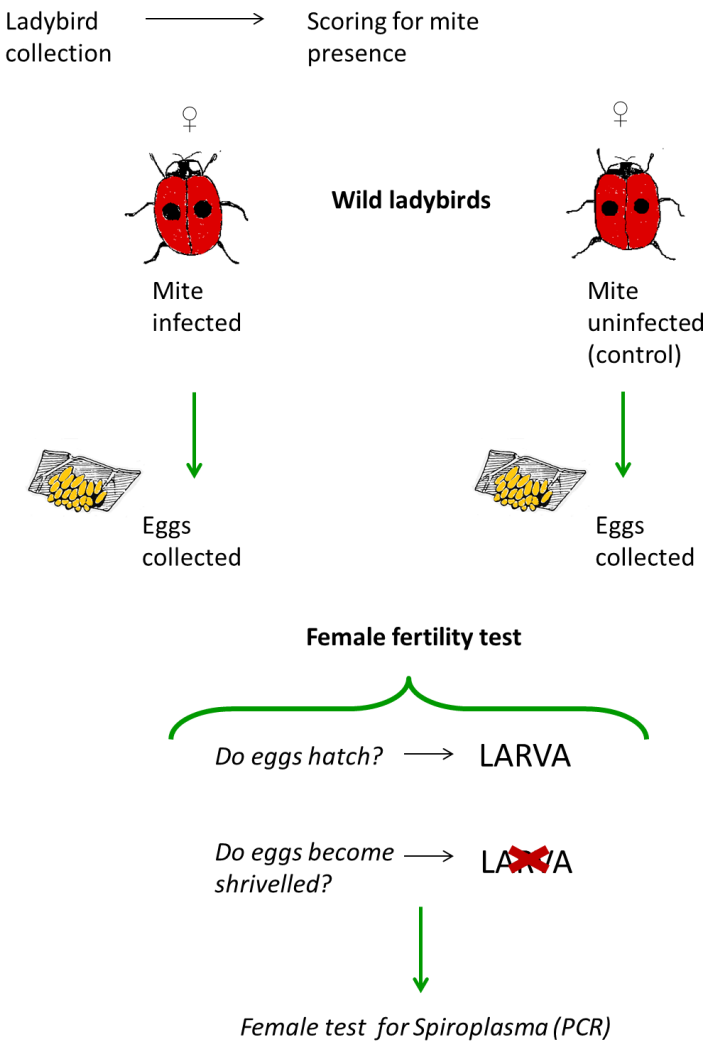


Figure 5.3: Experimental design: *Spiroplasma* effect on wild ladybird fertility.

5.2.2.2 Laboratory study of impact of Spiroplasma on ladybird fertility

The impact of *Spiroplasma* infection on the incubation period of mite-induced sterility was measured under laboratory conditions. Laboratory-reared *Spiroplasma*-infected (*N*=13) and uninfected (*N*=10) beetles were infected with mites through mating to infected males. Fertility of control females without mite infection was also recorded (4 *Spiroplasma* +ve and 12 *Spiroplasma* –ve).

All females were allowed to lay eggs and the fertility of these females was monitored daily. If a female laid eggs, the number of laid eggs was recorded. During the experiment all females were allowed to remate to uninfected males weekly to ensure there was no sperm depletion. The eggs were kept in the incubator at 22˚C, and egg development recorded. Females were deemed to be infertile when the eggs laid displayed the characteristic 'shrivelled' appearance within 48 hours of being laid. The day on which this occurred was then defined as the incubation period of the mite on that female (Figure 5.4). Difference in incubation period between *Spiroplasma* +ve and *Spiroplasma* –ve female ladybirds was then tested.

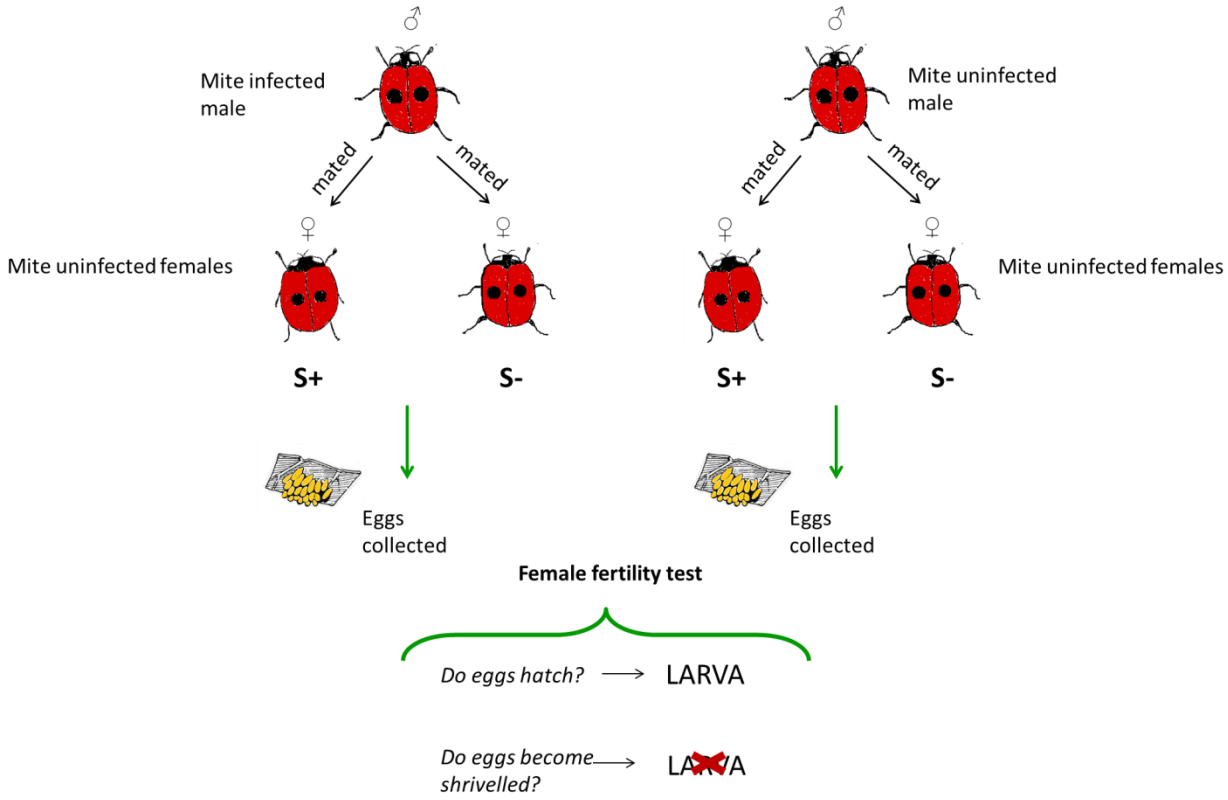


Figure 5.4: Experimental design: *Spiroplasma* effect on laboratory bred ladybird fertility.

5.2.3 Effects of Spiroplasma on longevity of overwintered ladybirds in the presence/absence of mites

Overwintered *Adalia bipunctata* were collected between the 21st and 24th of May 2013 from Stockholm and transferred individually in Eppendorf tubes to the laboratory. Ladybirds were then scored for sex and mite presence as previously described. These ladybirds were established in populations of 10 ladybirds (7 females and 3 males reflecting the sex ratio where male-killing bacteria is present) in a large Petri dish Sarstedt™ (140mm diameter, 20mm depth) (Figure 5.5). The aim was to establish eight populations in which the mite was absent (Figure 5.6), and eight in which mite was present initially in 1-3 infected individuals (females and/or males), reflecting mite prevalence early in the reproductive season. Within these 'mite infected' populations, infected individuals were marked with Tipp-Ex such that new infections could be distinguished (Figure 5.7). However, misscoring of a single ladybird in a control population led to one mite free population entering a mite epidemic, such that in effect seven uninfected with mites and nine mite infected populations were established. It is also important to mention that using Tipp-Ex creates a confounding variable in the study because this marking treatment might have affected the mating success of those marked ladybirds.

Figure 5.5: The ladybird populations in large Petri dishes (10 ladybirds per one Petri dish).

Each population was kept in the incubator at 20˚C for 20L:4D (20 hours of light and 4 hours of night when the temperature decreased to 10˚C) and fed aphids daily where available and artificial food on all days. Ladybirds which died were scored for sex and mite presence and all the information plus the date of death were recorded. Dead females were tested for *Spiroplasma* presence using methods presented in Chapter 2.

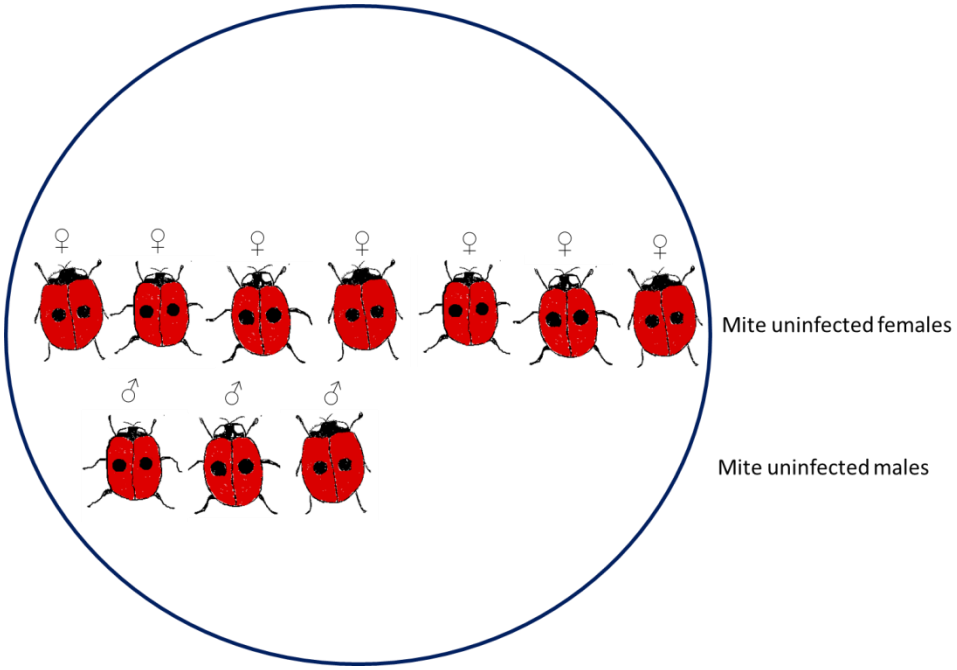


Figure 5.6: An example of experimental ladybird population without mite infected individuals (control).

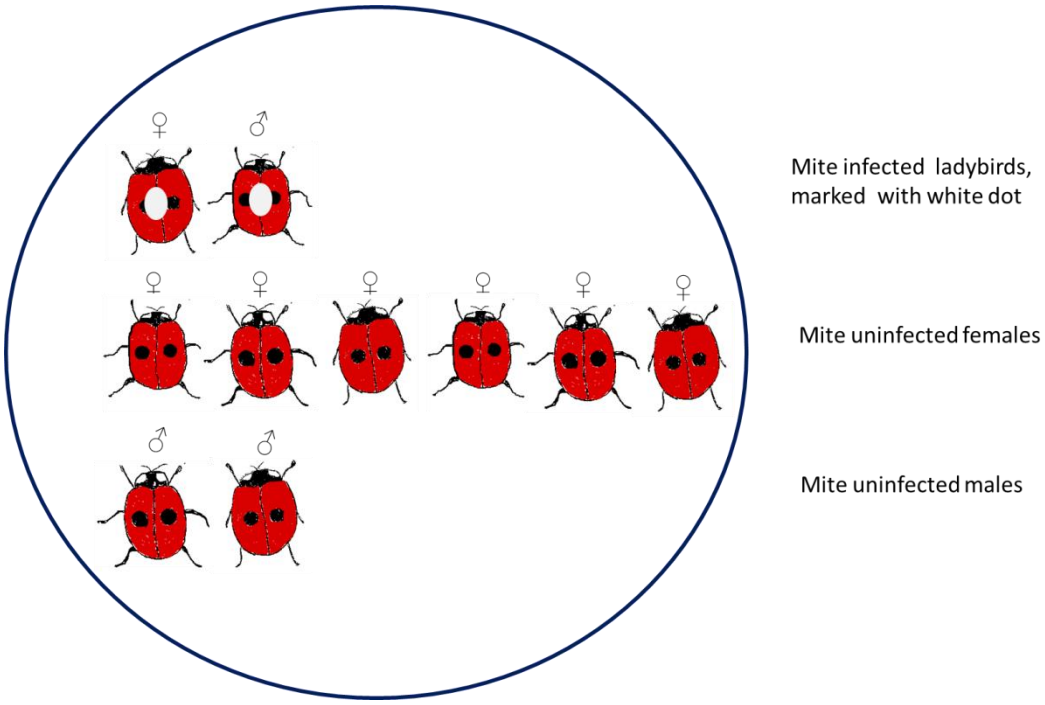


Figure 5.7: An example of experimental ladybird population with 1-3 individuals (females and/or males) infected with mite.

Statistical analysis

Spiroplasma and/or mite impact on the longevity of ladybirds and the variation in beetle death rate was assessed using a Cox Proportional-Hazard Regression model for survival. This statistical analysis tests the relationship between the survival of an individual and several explanatory variables. Survival analysis involves the modelling of time between entry to a study and subsequent event data, with ladybird death being considered the 'event' modelled against time. *Spiroplasma* and mite presence/absence were used as factors. Cox models were created using the Coxph function in R, and model fit compared through Likelihood Ratio tests (LRTs) with serial deletion of factors, and establishing if deletion of a factor significantly reduced model fit. Factors examined included interactions between factors *Spiroplasma* and mite, *Spiroplasma*, mite.

5.3 Results

5.3.1 Does the symbiont stop the host from acquiring and transmitting the mite?

5.3.1.1 Are Spiroplasma-infected ladybirds less likely to carry mites in the wild?

Mite infection status in field collected female ladybirds was not associated with *Spiroplasma* infection in Gävle or Malmö in 2011 (Table 5.1). A GLM model fit using binomial errors was not significantly affected when the term '*Spiroplasma* infection status' was removed (deviance associated with factor '*Spiroplasma* infection status' =0.95; df=1, AIC=32.009, LRT=0.6329 *P*=0.426). 'Sample collection' was associated with heterogeneity in this analysis (deviance associated with factor =13.338; df=2, AIC=42.397, LRT=13.02, *P*=0.0015). This reflects the epidemic spread of the mite over time.

In a similar fashion, ladybird samples from 2012 and 2013 from Stockholm showed no evidence of an association between *Spiroplasma* and the mite (Table 5.2 and 5.3). In both cases, city centre sites were treated together, as were suburban sites. In both 2012 and 2013, GLM model fit was not significantly affected when the term '*Spiroplasma* infection status' was removed (For 2012: deviance associated with factor '*Spiroplasma* infection status' =2.748; df=1, AIC=45.16, LRT=0.151, *P*=0.70; For 2013: deviance =2.09; df=1, AIC=30.90, LRT=0.473, *P*=0.49). 'Sample collection' was associated with heterogeneity in this analysis (For 2012: deviance =44.0; df=3, AIC=82.41, LRT=41.4, *P*<0.0001; For 2013 deviance =16.93; df=3, AIC=41.4, LRT=15.31, *P*=0.0015). This heterogeneity was largely associated with different times of collection (May vs August), as expected for a parasite with epidemic spread.

In contrast, an association between *Spiroplasma* and mite presence was observed in Stockholm in the collection from 2011 (Table 5.4). A GLM model fit using binomial errors was analysed to determine the effect of *Spiroplasma* on mite presence in ladybird populations within Stockholm. There was no *Spiroplasma*-site interaction term associated with mite presence, therefore it was not conserved in the model. However, both of the factors 'site' and '*Spiroplasma*' contributed to variance in mite

prevalence (‘*Spiroplasma*’: deviance associated with factor ‘infection status’ =15.69; df=1, AIC=53.25, LRT=8.72, *P*= 0.0031; ‘Site’: deviance associated with factor =52.4; df=2 AIC=83.9; LRT=45.46, *P*<0.00001). Deviance associated with site was expected from known temporal and spatial heterogeneity of mite prevalence (Ryder *et al.*, 2013). *Spiroplasma*-infected individuals were more likely to harbour mites across the 2011 samples.

Table 5.1: Mite and *Spiroplasma* infection status of *A. bipunctata* females collected during epidemic spread in Swedish cities: a) Gävle June 2011; b) Gävle July 2011; c) Malmö June 2011.

a) Gävle - June 2011

	Mite	
	Present	Absent
Spiroplasma positive	13	36
Spiroplasma negative	18	49

b) Gävle - July 2011

	Mite	
	Present	Absent
Spiroplasma positive	10	20
Spiroplasma negative	11	15

c) Malmö - June 2011

	Mite	
	Present	Absent
Spiroplasma positive	20	24
Spiroplasma negative	41	38

Table 5.2: Mite and *Spiroplasma* infection status of *A. bipunctata* females collected in different part of Stockholm in May and August 2012: a) City centre: Södermalm and Valhallavägen; b) Suburbs: Hägerstensåsen and Tensta.

a) City centre of Stockholm

Södermalm May 2012	Mite	
	Present	Absent
Spiroplasma positive	13	38
Spiroplasma negative	15	46

Valhallavägen May 2012	Mite	
	Present	Absent
Spiroplasma positive	8	48
Spiroplasma negative	15	74

Södermalm August 2012	Mite	
	Present	Absent
Spiroplasma positive	27	44
Spiroplasma negative	31	73

Valhallavägen August 2012	Mite	
	Present	Absent
Spiroplasma positive	14	11
Spiroplasma negative	34	18

b) Suburbs of Stockholm

Hägerstensåsen May 2012	Mite	
	Present	Absent
Spiroplasma positive	6	4
Spiroplasma negative	30	40

Tensta May 2012	Mite	
	Present	Absent
Spiroplasma positive	2	1
Spiroplasma negative	1	9

Hägerstensåsen August 2012	Mite	
	Present	Absent
Spiroplasma positive	1	19
Spiroplasma negative	0	7

Tensta August 2012	Mite	
	Present	Absent
Spiroplasma positive	2	1
Spiroplasma negative	7	23

Table 5.3: Mite and *Spiroplasma* infection status of *A. bipunctata* females collected in different part of Stockholm in May and August 2013: a) City centre: Södermalm and Valhallavägen; b) Suburbs: Hägerstensåsen and Kista.

a) City centre of Stockholm

Södermalm May 2013	Mite	
	Present	Absent
Spiroplasma positive	0	2
Spiroplasma negative	6	5

Valhallavägen May 2013	Mite	
	Present	Absent
Spiroplasma positive	1	6
Spiroplasma negative	0	10

Södermalm August 2013	Mite	
	Present	Absent
Spiroplasma positive	2	23
Spiroplasma negative	6	55

Valhallavägen August 2013	Mite	
	Present	Absent
Spiroplasma positive	0	18
Spiroplasma negative	1	64

b) Suburbs of Stockholm

Kista May 2013	Mite	
	Present	Absent
Spiroplasma positive	0	2
Spiroplasma negative	0	3

Hägerstensåsen May 2013	Mite	
	Present	Absent
Spiroplasma positive	1	20
Spiroplasma negative	0	7

Kista August 2013	Mite	
	Present	Absent
Spiroplasma positive	8	38
Spiroplasma negative	5	20

Hägerstensåsen August 2013	Mite	
	Present	Absent
Spiroplasma positive	0	1
Spiroplasma negative	0	1

Table 5.4: Mite and *Spiroplasma* infection status of *A. bipunctata* females collected during epidemic spread in June and July 2011 in different part of Stockholm: a) City centre: Södermalm and Valhallavägen; b) Suburbs: Hägerstensåsen, Hallonbergen and Tensta.

a) City centre of Stockholm

Södermalm July 2011	Mite	
	Present	Absent
Spiroplasma positive	17	0
Spiroplasma negative	15	4

Valhallavägen July 2011	Mite	
	Present	Absent
Spiroplasma positive	4	0
Spiroplasma negative	4	7

b) Suburbs of Stockholm

Hallonbergen June 2011	Mite	
	Present	Absent
Spiroplasma positive	15	25
Spiroplasma negative	3	8

Hägerstensåsen July 2011	Mite	
	Present	Absent
Spiroplasma positive	31	13
Spiroplasma negative	13	10

Tensta July 2011	Mite	
	Present	Absent
Spiroplasma positive	43	7
Spiroplasma negative	21	8

5.3.1.2 Does Spiroplasma produce resistance to mite infection in ladybirds in the laboratory?

The presence of symbiont-mediated protection was examined directly, through testing whether *Spiroplasma* affected a) mite transmission to female ladybirds; b) development of mite infection on beetles (Table 5.5). There was no evidence that *Spiroplasma* had an effect on:

a) Probability of female acquisition of the mite following mating (Fisher exact test: df=1, *P*>0.4);

b) Probability of retaining the mite (Fisher exact test: df=1, *P*>0.2);

c) Mortality of the host (Fisher exact test: df=1, *P*>0.66)

d) Latent period of infection (GLM: No significant variance associated with '*Spiroplasma* infection status': F=0.27, df=1, 38, *P*>0.85; Evidence of heterogeneity between blocks: F=14.46, df=1, 38, *P*>0.001).

There was also no evidence for an impact of *Spiroplasma* presence on the intensity of mite infection generated 30 days after initial infection. Results revealed that there was no evidence of an association between *Spiroplasma* presence and intensity of mite infection (mean number of reproducing mites on *Spiroplasma* positive (S+) females =7.08 +/- 1.30; mean number of reproducing mites on *Spiroplasma* negative (S-) females =6.80 +/- 1.30; t=0.16, df=21, *P*>0.8).

Table 5.5: The impact of host sex and *Spiroplasma* infection status on acquisition, persistence and mite transmissions in Swedish *A. bipunctata*. Sample size is given in parentheses; errors for latent period are +/- one standard error (adapted from Ryder *et al.*, 2014).

Metric	Block	Males	Females	
			Spiroplasma positive (S+)	*Spiroplasma* negative (S-)
Proportion of individuals acquiring infection following copulation	1	0.94 (54)	0.941 (17)	0.875 (8)
	2		0.823 (17)	0.75 (16)
Recovery rate of infected individuals in first 20 days	1	0 (34)	0 (16)	0 (7)
	2		0 (16)	0.071 (14)
Latent period of infection (Days, +/- 1 s.e.)	1	21.45 +/- 0.62 (20)	21.58 +/- 0.63 (12)	22.33 +/- 0.95 (6)
	2		24.50 +/- 0.59 (13)	23.70 +/- 0.42 (10)
Proportion of ladybirds dying in 20 days post-infection	1	0.382 (34)	0.1875 (16)	0.25 (8)
	2		0.133 (15)	0.214 (4)

5.3.2 Does Spiroplasma protect the host against mite-induced infertility?

5.3.2.1 Are Spiroplasma-infected females in the wild fertile despite mite infection?

All female *A. bipunctata* collected from the field were infertile where the mite was present, notwithstanding *Spiroplasma* infection status. All females uninfected with the mite females were, in contrast, fertile (Table 5.6). Statistical analysis using GLM with binomial errors found that there was no evidence for mite-*Spiroplasma* interaction term with respect to host fertility (P=1.0). As expected, when the interaction term was removed from the model, an effect of mite on fertility was observed, with no impact of the *Spiroplasma* (*Spiroplasma*: deviance associated with factor '*Spiroplasma* infection status' =0, df=1, AIC=4, LRT=0, P= 1; Mite: deviance associated with factor =21.209, df=1, AIC=25.21, LRT=21.21, P<0.00001).

Table 5.6: Fertility of field collected *A. bipunctata* females in the presence/absence of mite, partitioned by *Spiroplasma* presence/absence.

Female	Proportion fertile females mite present (*N*)	Proportion of fertile females mite absent (*N*)
Spiroplasma +	0.0 (3)	1.0 (8)
Spiroplasma -	0.0 (3)	1.0 (3)

5.3.2.2 Impact of Spiroplasma on host fertility in laboratory bred ladybirds

Female ladybirds infected with the mite became infertile in all cases, notwithstanding *Spiroplasma* infection status. In contrast, fertility was high in mite-uninfected controls (Table 5.7). Statistical analysis using GLM with binomial errors found that there was no evidence for a mite-*Spiroplasma* interaction term with respect to host fertility (Test for presence of an interaction term P=1.0). As expected, when the interaction term was dropped from the model, an effect of mite on fertility was observed, with no impact of the *Spiroplasma* (*Spiroplasma*: deviance associated with factor '*Spiroplasma* infection status' =0.597, df=1, AIC=6.512, LRT=0.597, P=0.42; Mite: deviance associated with mite presence/absence =42.16, df=1, AIC=48.08, LRT=42.16, P<0.00001). The incubation period (the time before female ladybirds became infertile in the presence of the mite) did not differ between *Spiroplasma*-infected and *Spiroplasma*-uninfected ladybirds (Figure 5.8) (t-test, df=21, P=0.23).

Table 5.7: Impact of the mite on fertility in the presence/absence of *Spiroplasma* in laboratory reared ladybirds.

Mite present?	*Spiroplasma*	*N*	Proportion of fertile female?
Yes	S+	13	0.0
Yes	S-	10	0.0
No	S+	4	1.0
No	S-	12	0.9

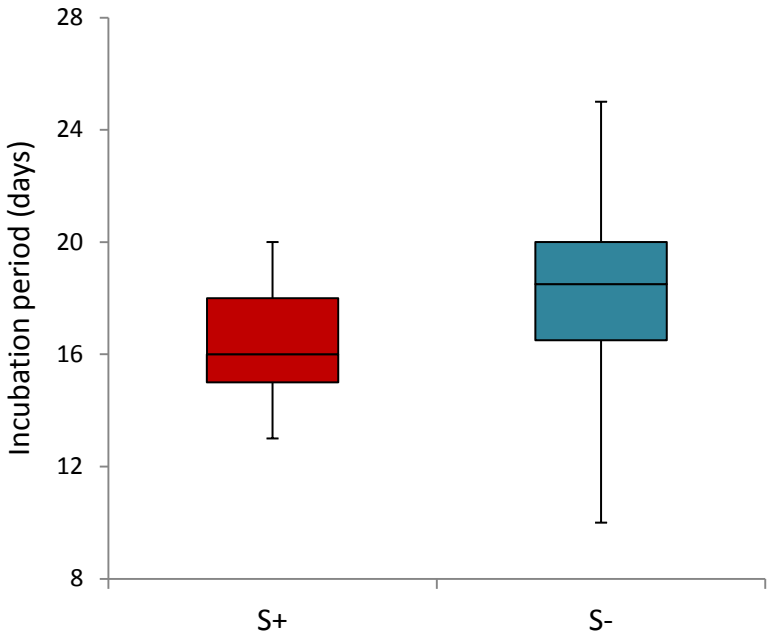


Figure 5.8: The incubation period of the mite on *Spiroplasma*-infected (red colour●) (S+) and *Spiroplasma*-uninfected (blue colour●) (S-) ladybirds. Sample size: *N*=13 and *N*=10 respectively.

5.3.3 Longevity of overwintered ladybirds: effects of mite and Spiroplasma

The ladybird longevity experiment lasted 145 days beginning on 9th June 2013 and finishing on 1st November 2013. Mating was observed commonly in all populations (with and without mites). Nearly all individuals in the 'mite infected' populations carried mites at the point of death, indicating the mite had undergone epidemic spread through these populations as would occur in natural populations (Webberley *et al.*, 2006a; Ryder *et al.*, 2013) (Table 5.8).

Table 5.8: Proportion of ladybirds which carried mites at the point of death.

Population	Mite present in the population	Proportion of females carried mites at the point of death	Proportion of males carried mites at the point of death
INF1	+	1.0 (7)	1.0 (3)
INF2	+	1.0 (7)	1.0 (3)
INF3	+	1.0 (6)	1.0 (3)
INF4	+	1.0 (7)	1.0 (3)
INF5	+	1.0 (6)	1.0 (3)
INF6	+	1.0 (7)	1.0 (3)
INF7	+	0.86 (7)	1.0 (3)
INF8	+	1.0 (7)	0.33 (3)
C2	+	0.67 (6)	1.0 (3)
C1	-	0.0 (6)	0.0 (3)
C3	-	0.0 (7)	0.0 (2)
C4	-	0.0 (7)	0.0 (2)
C5	-	0.0 (4)	0.0 (2)
C6	-	0.0 (7)	0.0 (2)
C7	-	0.0 (6)	0.0 (3)
C8	-	0.0 (7)	0.0 (3)

5.3.3.1 Assessing impact of Spiroplasma and mite infection on female longevity

The survivorship of the four classes of female: mite-infected, mite-uninfected with and without *Spiroplasma*, is given in Figure 5.9. Analysis indicates an interaction term between mite and *Spiroplasma* presence associated with longevity (LRT

comparing model fit with and without interaction term: χ^2=4.61, df=1, *P*=0.03). Model fit was not significantly impacted by dropping either the factor *Spiroplasma* or the factor *mite* individually from the model (LRT comparing model fit with and without '*Spiroplasma*' factor: χ^2=3.58, df=1, *P*=0.056; LRT Model with and without 'mite' factor: χ^2=2.79, df=1, *P*=0.09). Overall, the data indicate that females *A. bipunctata* that carry neither mites nor *Spiroplasma* die more rapidly than those which carry either mites, *Spiroplasma* or both mites and *Spiroplasma*.

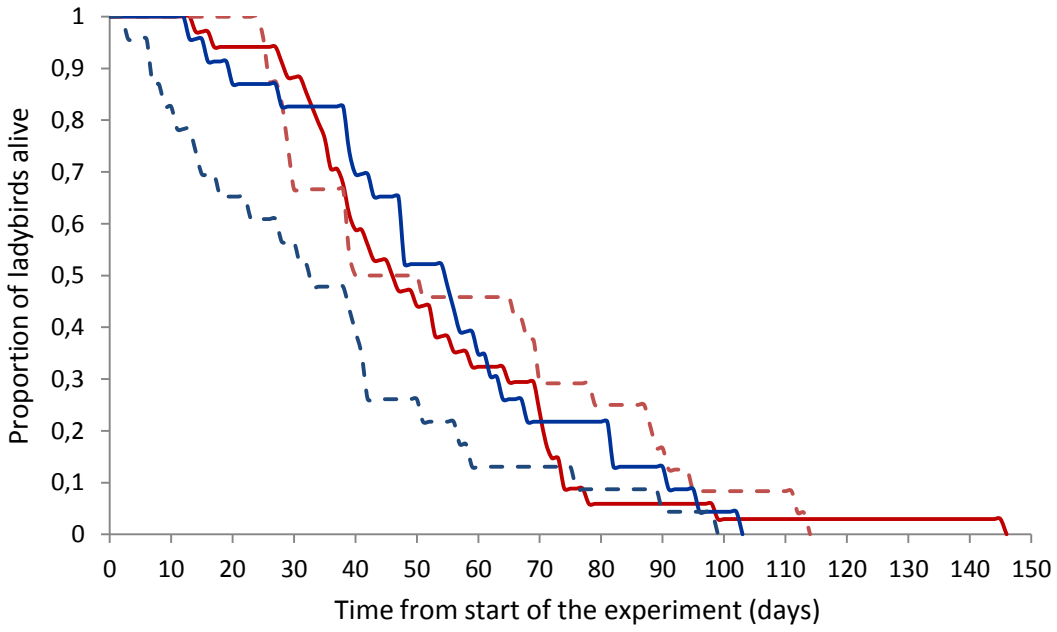


Figure 5.9: Survival of *A. bipunctata* females: individuals infected with *Spiroplasma* and the mite, *N*=34 (solid red line —); females infected with *Spiroplasma* but not with mites, *N*=24 (dash red line ---); individuals not infected with *Spiroplasma* but infected with the mite, *N*=23 (solid blue line —); females not infected with *Spiroplasma* and not infected with mites *N*=23 (dash blue line ---).

5.4 Discussion

It is increasing realised that heritable symbiont infection may provide protection for its host against attack by another parasite (symbiont-mediated protection) (Oliver *et al.*, 2003; Ferrari *et al.*, 2004). Further, symbionts may affect longevity of their host (Elnagdy *et al.*, 2013), potentially affecting the epidemiology of other infections. These observations motivated this direct examination of impacts of *Spiroplasma* on two-spot ladybird tendency to acquire, and be affected by, the mite *C. hippodamiae*.

There was no evidence for *Spiroplasma* having impact on the ladybird host likelihood of acquiring mite infection or on the development of any infection that arose. Laboratory experiments indicated that there were no differences between *Spiroplasma*-infected and uninfected females in the tendency to acquire mites during copulation with an infectious male. There were also no differences in mite development and latent period on these ladybirds. There was also no evidence that *Spiroplasma* alters the infertility impact of the mite. Both wild ladybirds and laboratory ladybirds, were made infertile by the mite irrespective of *Spiroplasma* infection status. Further, in the laboratory, *Spiroplasma* does not alter the incubation period of the host (time until their host becomes infertile).

This data indicates that the *Spiroplasma* frequency will not be altered by the presence of the mite (as expected if it rescued mite-induced sterility). Further to this, the presence of *Spiroplasma* will not affect epidemic spread of the mite through direct effects on host susceptibility. It can also be concluded that a phenotype that would be very useful for both symbiont and host (protection) does not necessarily evolve, despite the presence of strong selection in this system for protective function.

Whilst the laboratory data on mite-*Spiroplasma* interactions were clear, field data examining mite/*Spiroplasma* revealed some contrasts. In four cases, there was no association between *Spiroplasma* and mite in field ladybird populations from Gävle/Malmö and from Stockholm 2012 and 2013. However, in one case

(Stockholm 2011) it was observed that females infected with *Spiroplasma* were more likely to carry the mite. This tendency was observed in both city centre and suburban populations.

There are three possible causes which might explain this association between mite and *Spiroplasma* within a population:

a) The mite itself can carry *Spiroplasma* infection, such that mite-infected ladybirds appear *Spiroplasma* positive in a PCR assay.

b) The *Spiroplasma* makes female ladybirds more likely to retain mite infection or less likely to die following mite infection.

c) *Spiroplasma*-infected female ladybirds have higher exposure to the mite during spring.

Of these, the first is unlikely. An expectation is that female hosts would be more likely to score positive for *Spiroplasma* later in the season, following epidemic spread of the mite, and this is not observed. It also predicts males would commonly score positive for *Spiroplasma* if they were mite infected, and they do not. It is also hard to imagine how this would occur just in Stockholm and just in one year. The second explanation is not supported by above experiments, which indicates no impact of *Spiroplasma* on the likelihood of Stockholm *Adalia* acquiring or retaining mite infection. The third explanation would require *Spiroplasma*-infected females to have higher mating rate, but this would be restricted to Stockholm in 2011. This cannot be ruled out, but would be an unusual sporadic effect. Altogether, the source of the association in Stockholm 2011 remains enigmatic.

The second set of experiments examined impacts of *Spiroplasma* on longevity. Previous study indicated *Spiroplasma* presence can be beneficial for adult female longevity (Elnagdy *et al*. 2013). The experiment conducted differed from Elgnady *et al.* in utilizing field-collected beetles that had emerged from the stress of diapause, and examining longevity both in the presence and in absence of the mite (the previous study was mite-free). In addition, high nutrition supply to the ladybird (alive aphids) was maintained wherever possible, contrasting with the use of

artificial diet in Elnagdy *et al.* (2013). The results are in part consistent with the previous study, in that *Spiroplasma*-infected females survived around three weeks longer than *Spiroplasma*-uninfected females in the absence of the mite. The effect was, however, modest in magnitude compared to the three-fold increase in longevity observed by Elngady *et al.* Surprisingly, the impact of *Spiroplasma* on longevity disappeared in the presence of mite infection, with *Spiroplasma*-uninfected beetles showing similar longevity to *Spiroplasma*-infected ones in the populations where the mite was present.

The longevity study produces a variety of questions. First, is the effect observed repeatable? The data presented indicate statistical support for the presence of an interaction term at P=0.03, and warrants repeat to ensure this is not a 'false positive' result. Second, if the effect is robust, why is the effect of *Spiroplasma* on longevity in the absence of the mite much smaller than observed by Elgnady *et al.* (2013). One possibility here is that Elgnady *et al.* fed ladybirds artificial food after 40 days, compared to aphid feeding throughout my experiment. Commonly, ladybird longevity is prolonged on artificial food (personal observations), which may explain the magnitude of difference. Third, why is there an interaction term between *Spiroplasma* and mite and longevity? One hypothesis here is that both act through the same physiological pathway to make long lived, but ultimately less fertile hosts.

The impact of longevity increases associated with mite/*Spiroplasma* on mite epidemiology also warrants consideration. It is notable that host longevity is highest in the presence of *Spiroplasma* or mite. These conditions promote contact and transmission between cohorts, and thus allow mite persistence (Chapter 3). Indeed, the ability of the mite itself to extend longevity (if repeatable) is significant, as this would equate to the mite increasing the likelihood of the host individual transmitting the mite into the next cohort. This would be especially important in locations where the *Spiroplasma* (which itself appears to increase longevity) is rare or absent, such as Poland. As such, increased longevity associated with the mite would represent an adaptive trait for the mite, potentially gained through fertility reduction of the host.

Chapter 6: Assessing the impact of symbiont-induced sex ratio bias on the dynamics of sexually transmitted infections in the two-spot ladybird *Adalia bipunctata*

1. Theory predicts that the epidemiology of sexually transmitted infections (STIs) will be affected by any factor that alters the mating biology of its host. In insects, the presence of symbionts that distort the host sex ratio may modify population sex ratio by altering male and female encounter rates and thus STI dynamics.
2. The two-spot ladybird *Adalia bipunctata* is a host for both a heritable male-killing bacteria *Spiroplasma* and a sexually transmitted mite *Coccipolipus hippodamiae*. In some populations, the male-killing symbiont reaches 70% prevalence, causing strongly female-biased population sex ratios.
3. Laboratory experiments were used to investigate how the host population sex ratio bias produced by a bacterial symbiont will have an impact on STI epidemiology through changing of host's mating system. I compared mating biology and STI transmission under 1:1 and 4:1 population sex ratio, which are equivalent to non-male-killer presence and 75% male-killer prevalence.
4. I observed that males are able to mate with enough females to maintain high female mating rate under strongly female-biased population sex ratio. Further, I observed that males inhibit female remating less when males mate commonly, suggesting female mating rate may in fact be raised in female-biased populations.
5. Female partners of recently mated to mite-infected males were less likely to establish a mature infection than females mated to mite-infected males without a recent history of mating. This was associated by a lower intensity of initial infection where males mated commonly, which then did not persist.
6. I conclude that the presence of male-killing bacteria is likely to have indirect impacts on female fertility, mediated through the STI.

This chapter, in modified form, has been accepted for publication:
Pastok D., Atkinson, D. and Hurst, G. D. D. "Assessing the impact of male-killing bacteria on the spread of a sexually transmitted infection." *Animal Behaviour*

6.1 Introduction

Sexually transmitted infections are common in animal species where at least one sex is promiscuous (Lockhart *et al.,* 1996; Knell and Webberley, 2004). The dynamics of STIs are often considered as distinct from that of other infections, as transmission rates are related to the host mating system, rather than more general patterns of movement. Sexually transmitted infection epidemiology depends both on the mean number of partners with which an individual mates (promiscuity), the variance in mating success amongst individuals of a particular sex, and the contact structure of matings (i.e. whether more promiscuous individuals tend to pair together) (Lockhart *et al.,* 1996; Thrall *et al.,* 1997; Thrall *et al.* 2000; Ashby and Gupta, 2013). For instance, high variance in male reproductive success associated with male-male competition for mates produces female-biased epidemics of STIs (Nahrung and Allen, 2004; Ashby and Gupta, 2013).

In order to understand how STI epidemiology varies, therefore, we must understand the factors driving the variation of mating system. One ecological factor known to affect mating systems is a population sex ratio (Rankin and Kokko, 2007). Many insects carry sex ratio distorting microbes or selfish genetic elements that create strongly female-biased populations and these are known to alter mating systems (Charlat *et al.*, 2007; Hurst *et al.,* 1997; Jiggins *et al.,* 2000). However, the impact of sex ratio bias on STI epidemiology has been poorly researched.

Theoretical studies suggested that in a 'normal' population with a 1:1 sex ratio, the mean mating rate of male and female individuals is the same, whereas the STI epidemics are female-biased. In this population, alpha males ('super-spreaders' (Ashby and Gupta, 2013)), which are more 'attractive' and 'fitter', attain the most matings and are therefore more likely to acquire infection. In contrast beta males are less active and mate less, decreasing exposure to STI parasites and reducing the probability of infection. In this type of population females choose and mate mainly with alpha males. When all females in that population have contact with alpha males, they are expected to become infected faster than males and a female-biased

STI epidemic take place. Moreover, even if beta males are common, the population is also characterized by a female-biased epidemic (Ashby and Gupta, 2013).

Nahrung *et al.* (2007) has observed the above situation in the population of a sub-tropical eucalypt beetle *Chrysopharta cloelia*. The population is characterized by having 1:1 sex ratio. *Chrysopharta* beetles are a host for a sexually transmitted parasite mite *Parobia captivus (*from the family *Podapolipidae*). Within the host population, large male beetles mate more frequently, and therefore are more likely to become infected. While small males are less successful, so their probabilities to become infected are reduced. Females are usually larger than males and they often choose larger males, increasing the chance for females to be infected. These factors foster a female-biased epidemic.

However, when a sex ratio bias is present in this population, the dynamic of an STI might look different. In female-biased populations, males on average mate more often than females, therefore their exposure to infected partners is higher. This creates the prediction that STI epidemics should switch to being male-biased under female-biased population sex ratio (Hurst *et al.,* 1997; Ryder *et al.,* 2014).

Recent studies have established this pattern in populations of the two-spot ladybird, *Adalia bipunctata* (Webberley *et al.,* 2006a; Ryder *et al.,* 2014). For instance, the observations on the ladybird populations from Stockholm (Sweden) revealed that even 70% of females carry the *Spiroplasma* infection, producing a sex ratio bias in the population of four females per one male. In this population, males mate more commonly than females and male mating rate is four times higher than female mating rate. More frequent male mating opportunities result in higher exposure of ladybird males to the STI. In this high *Spiroplasma* prevalence population a male-biased epidemic of the STI occurs approximately 2 weeks before that observed on females (Ryder *et al.*, 2014) (Exemplar data: Figure 6.1). In contrast, in Toruń (Poland) where the ladybird populations are 1:1 sex ratio (*Spiroplasma* is rare or absent), the STI epidemic spread occurs at similar time on females and male host (Webberley *et al.*, 2006a; Ryder *et al.,* 2014).

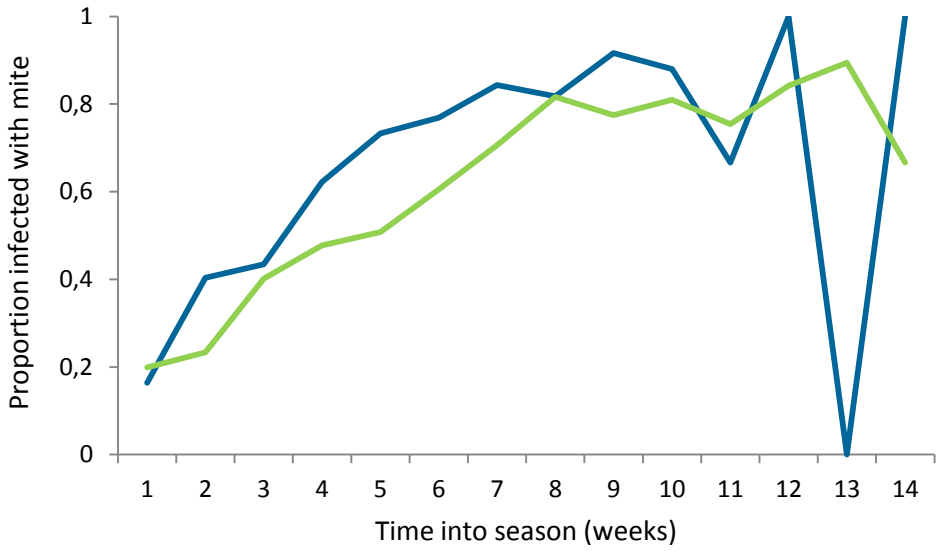


Figure 6.1: The prevalence of mite *Coccipolipus hippodamiae* on the overwintered adult cohort of males (●) and females (●) *A. bipunctata* in suburb populations of Stockholm (Sweden) from May (week 1) to August (week 14) in 2010 (adapted from Ryder *et al.,* 2014).

Whilst the gross impact of population sex ratio bias on STI dynamics (male-biased epidemics) is evident in *Adalia*, our understanding of how STI epidemiology is altered by male-killing bacteria/sex ratio skew is very basic. Sex ratio skew produces obvious impacts on the relative mating rate of male and female, predicting a male-biased epidemic. However, sex ratio biases may additionally have an impact on the speed of STI spread, and this impact may depend on how a female-biased sex ratio has an effect on the absolute mating rate of both sexes. Currently the effect remains unknown, demanding further research and observations.

Based on the theoretical prediction, two obvious scenarios are possible:

1) Male mating rate shows low plasticity, and female mating rate thus declines because female mating becomes limited by access to available males. This scenario is most likely where males invest heavily in paternal care, for instance through provision of a nutritious spermatophore. Constrains on male mating rate would create an epidemic, mildly slowed on male hosts (associated with lack of infection on female partners), and greatly slowed on females.

2) Male mating rate shows sufficient plasticity that female mating rate remains the same in female-biased populations, and male mating rate elevates the rate seen in a 1:1 population due to the increased availability of females. This would create an epidemic greatly accelerated on male host, and an epidemic in female hosts that was initially like that in a 1:1 population, but with later acceleration following the epidemic through male hosts.

Beyond this, it is also a possibility that female mating rate increases where males are rare. In *Hypolimnas bolina* butterflies, females remating rate is higher in female-biased populations. This is a result of males with high remating rate not transferring sufficient spermatophore material to inhibit their female partners from further remating (Charlat *et al.*, 2007). In ladybirds, males also inhibit female remating through a spermatophore that is transferred to a female partner during copulation, but in this case a female releases a spermatophore immediately after copulation and consumes it (Perry and Rowe, 2008). It is possible this resistance to remating associated with transfer of spermatophores is diminished when males mate frequently, and transfer either fewer or smaller spermatophores. If females remate more willingly in the presence of males with recently mate history, then we would predict that sex ratio skew would be associated with an STI epidemic that was also substantially accelerated on female hosts.

The epidemiology of STIs also depends on the chance of acquiring an infection during a sexual contact, and also the chance of acquiring an infection outside of copulation, for instance overwintering (Webberley and Hurst, 2002). In the ladybird system, new infections are produced by the transfer of motile larval mites. An adult mite on an infected host will produce a limited number of larvae each day (one mature adult mite lays 1-3 eggs per day; wild ladybird females carry 1-6 mature female mites) (Hurst *et al.*, 1995). Larval mite supply is enough to create very high transmission efficiency per copulation at low mating rates (Hurst *et al.*, 1995; Ryder *et al.*, 2014). However if a ladybird host mates very often, this may reduce a 'pool' of larval mites which are available for transfer, and it might reduce the probability of mite acquisition in the next copulation (Hurst *et al.,* 1995). Further, female

ladybirds that have mated recently (especially more common in the population with 1:1 sex ratio) may reject a partner in the following mating opportunities. These observations revealed that some females that rejected encounter mite infected males became infected. Therefore, rejection behaviours are partly protective, but that contact between male and female may be sufficient to allow for mite transfer. The study to test whether rejection behaviour protects against mite transmission was described in more details in Chapter 4.

The impact of sex ratio on mating biology and mite transmission is not easily measured in the field, as populations that differ in sex ratio also differ in a wide variety of other factors that affect mating rate (e.g. temperature or food sources) (Ryder *et al.*, 2013). Therefore, laboratory experiments were used to test the possible impact of a female-biased population sex ratio in *Adalia bipunctata* on the epidemiology of *C. hippodamiae*. I tested first whether a male that was offered females at 4x the 'natural' rate achieves 4x as many copulations, and whether a male that mates commonly was as likely to transfer mite infection to his partner. Second, I examined whether female willingness to remate is increased if males have a recent history of mating activity.

6.2 Materials and Methods

Adult two-spot ladybirds (*Adalia bipunctata*) were collected in Stockholm (Sweden) in August 2013 to produce experimental generations. All ladybirds were transported to the laboratory where they were scored for sex, mite presence and cohort. Mites were absent from all newly emerged ladybirds as they had no opportunities to mate with an overwintered cohort of adults, whereas nearly all collected older ladybirds (females and males) carried mites. Those older mite-infected females were used to infect some of the young adult males for the experimental generation. Therefore uninfected young males and mite infected old females, were paired and allowed to mate. The following day beetles were separated and males were checked for the presence of larval mite. Mite infection development was then monitored on the 7th, 14th, 19th day and daily onwards. Males used in the experiment were those that had developed 5-30 larval mites, implying a mature infection and had previously mated to uninfected females to reduce artefactual behaviour as a result of being separated from females (note, mite infection does not affect male mating success in this species; Webberley *et al.*, 2002). Females for experiments were uninfected in all cases, and either collected from the field as newly generated adults, or bred in the laboratory and matured for 20 days. Virgin females were used where possible. Where female re-use was necessary, the female had not mated in the previous 7 days, which restores female desire to mate to levels equivalent to virgin individuals (Haddrill *et al.*, 2007).

6.2.1 Can males maintain a high mating rate with increased exposure to females, and is mite transmission affected by male mating rate?

Sixty male ladybirds that were infectious with mites were mated three days prior to the experiment, and then offered four females in sequence in two mate exposure treatments. In the first treatment 'standard mating opportunity', males were offered an opportunity to mate every two days (in the morning), with four mating opportunities spread over 6 days. This mating rate (once every two days) was the

equivalent of that achieved by a male in a population with 1:1 sex ratio (Haddrill, 2008). In the second treatment 'frequent mating opportunity', males had females offered at four times the rate normally available. In this treatment males were offered females in the morning and in the evening for two days. All female partners used in this experiment were virgin and mite free (Figure 6.2).

Each mating opportunity lasted one hour, and the presence/absent of mating recorded. When mating occurred, ladybirds were placed in the incubator at 22˚C and allowed to finish mating, and mating duration recorded before separating ladybirds to individual Petri dishes. The recipient females were then maintained at 22˚C and the number of larval mites on the female was recorded 24 hours later. The development of these infections was then monitored through scoring mite infection again after 14 days, the time at which onward infection becomes possible (Ryder *et al.,* 2014) (Figure 6.3). The experiment was performed over three blocks with 10 'frequent mating opportunity' and 10 'standard mating opportunity' male ladybirds per block, for a total 30 males per treatment.

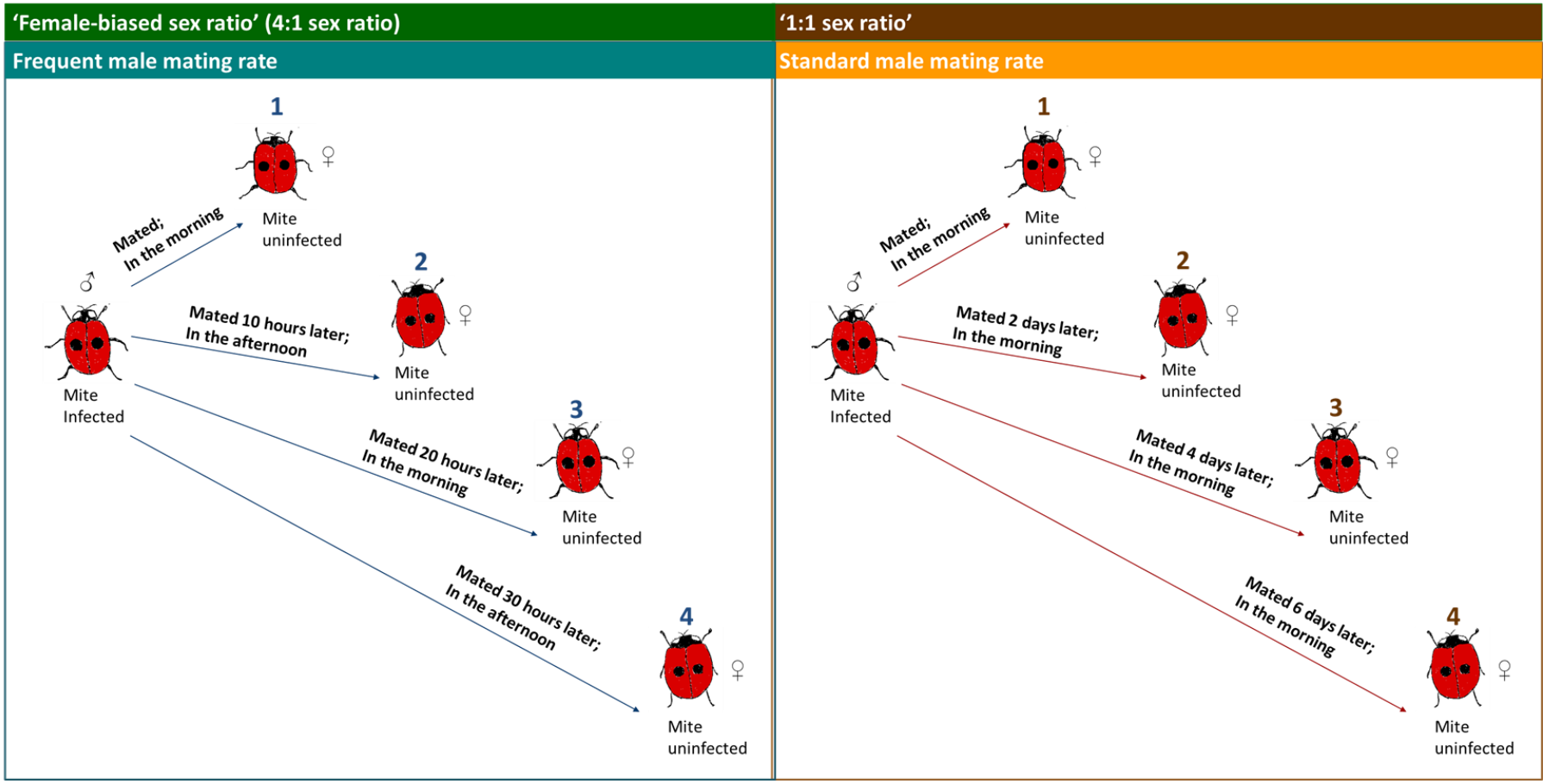


Figure 6.2: Experimental design: Testing if ladybird males can maintain high mating rate with increased exposure to females (the population with 4:1 sex ratio). The population with 1:1 sex ratio is a control.

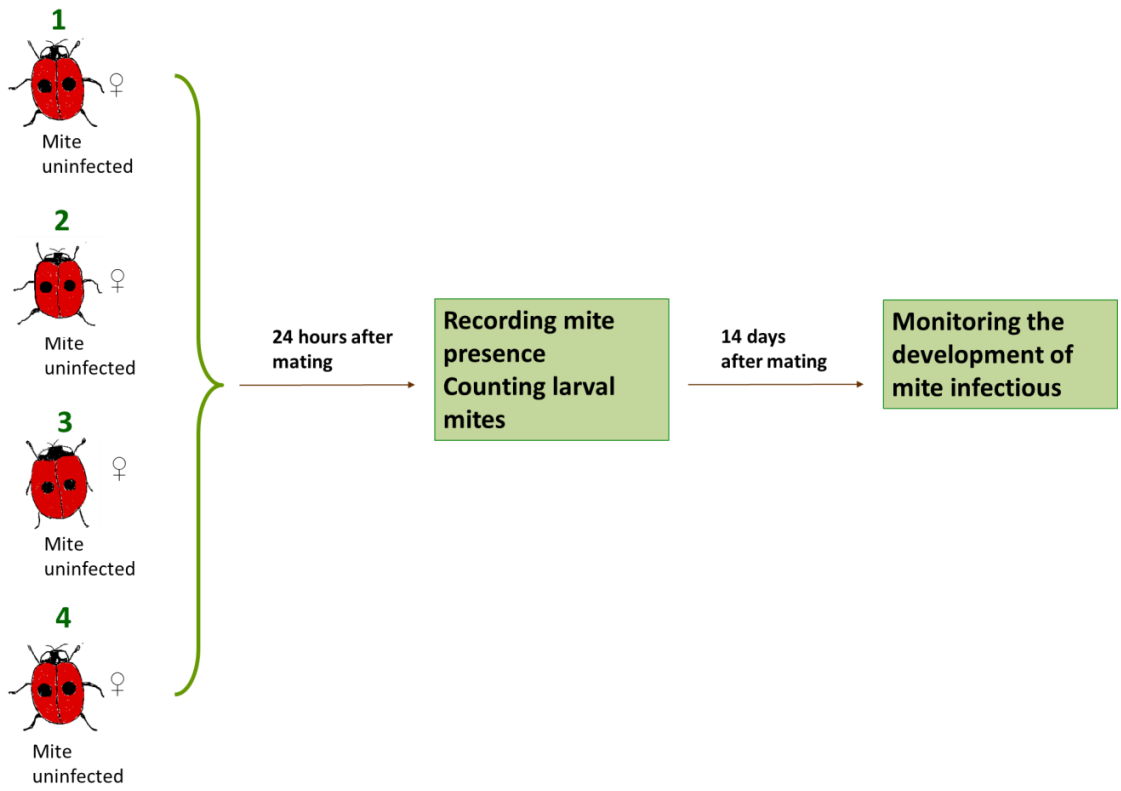


Figure 6.3: Experimental design: Testing if the mite transmission is affected by male mating rate in the population with 4:1 sex ratio and in the population with 1:1 sex ratio.

Statistical analysis

I first tested whether the probability of mating, duration of mating, and chance of mite transmission differed between treatment groups ('frequent mating opportunity'/'standard mating opportunity') for the first mating opportunity. Here, the expectation was that there should be no effect treatment as this interaction had the same history for both treatment groups. Following this, the impact of the factor 'mate exposure rate' on the outcomes of interactions 2, 3 and 4 was investigated:

a) Probability of mating. A GLM binomial logit model was used to examine whether a male successfully mated a female. Mating order (second, third or fourth female presented to him), and mate exposure rate ('frequent'/'standard') were used as the two predictive factors;

b) Mating duration. A GLM binomial logit model was used to examine the impact of factors mating rate, order of mating and interaction between them on the mating duration, whether it was long or short. For pairs which mated, the response variable mating duration was analysed similarly. This variable was not normally distributed, not transformable, and was split into long matings (>224 minutes) and short matings (<224 minutes) based on the median copulation duration during the first mating;

c) Transfer of mites. The analysis was performed to examine whether a successful mating produced a mature mite infection on the recipient female (presence/absence of mites 14 days after mating). First I examined whether the rate of exposure to mates ('frequent'/'standard' mating opportunity) had an effect on the probability of a female developing a mature mite infection (presence/absence of mites 14 days after mating) using a GLMM binomial logit model with male as a random factor. The impact of treatment, 'frequent'/'standard' mating opportunities, order of mating, and the interaction between these two on the response variable 'mite presence at day 14' was examined.

The process of infection was then split into its components: initial transmission occurring (response variable: presence of mites on day 2), the intensity of infection produced on initial transmission (response variable: high intensity of infection >5 larval mites and low intensity of infection 1-5 larval mites based on the bimodal intensity observed in the data), and progression of initially infected individuals to being infected.

The former pair of analyses was conducted using a binomial logit mixed model. The final analysis (progress of infection) used a Fisher exact test to investigate whether the initial intensity of mite infection (high/low number of larvae present at day 2) affected the probability of developing a mature infection (mites present at day 14).

6.2.2 Do females mate rapidly if they mated to a recently mated male?

Females were mated to two types of uninfected male ladybird, 'recently mated' and 'rested'. In the 'rested' treatment, the male had not mated in the previous 5 days. In the 'recently mated' treatment, the male had mated 1-3 hours previously. One hour after the first copulation, the response of the female to a second 'tester' male was then examined (non-mating females were discarded).The 'tester' male was well rested which means it didn't have mating opportunities in previous five days. The interaction between female and 'tester' male was observed for up to 15 minutes, and recorded interactions divided into three categories: a) No interaction during the period of observation; b) Rejection behaviours, when females rejected a male partner in first instance (see Webberley *et al.,* 2002); c) Acceptance, where a female accepted a partner straight away without any rejection behaviour (Figure 6.4).

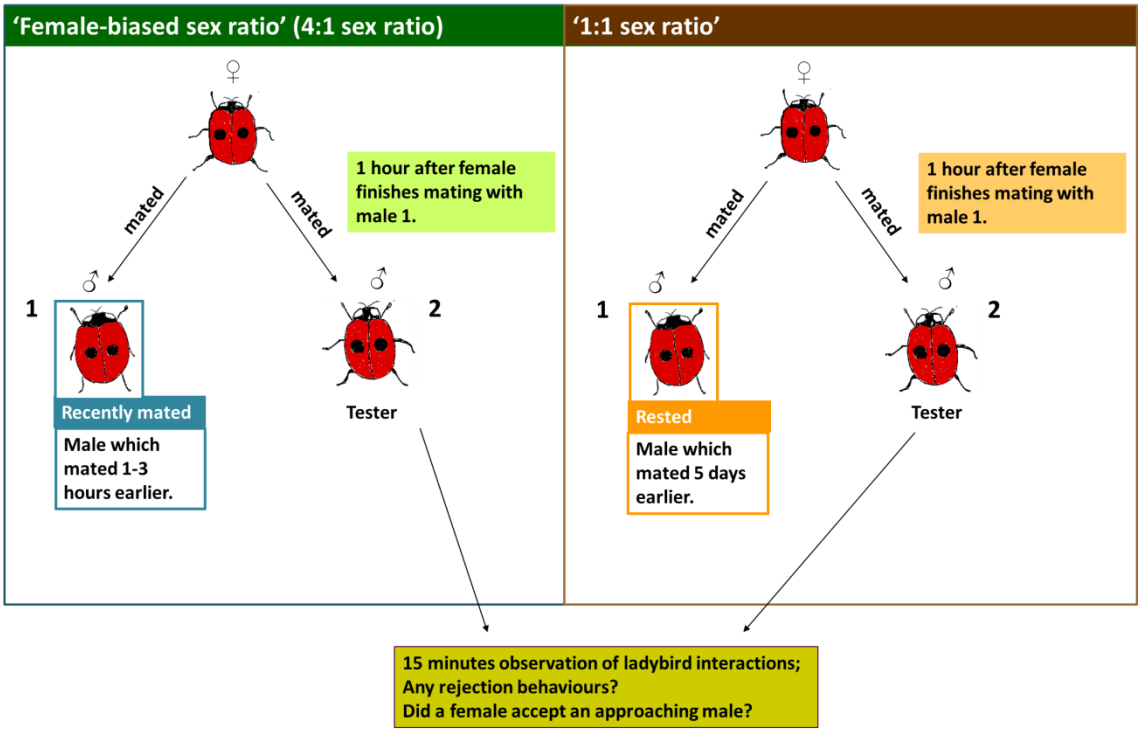


Figure 6.4: Experimental design: Investigating if females that had mated to a male with recent history of mating, were willing to remate.

Statistical analysis

The impact of first male partner mating status ('recently mated' vs 'rested'), on whether the female interacted with the second 'tester' male, was examined using general linear models with a binomial logit function. If ladybirds interacted, the impact of first male partner mating status upon whether they accepted/rejected the mating opportunity was also examined. Males were re-used in this experiment in different blocks (at least one week of rest), and male identity included as a random factor in analysis.

6.3 Results

6.3.1 Can males maintain a high mating rate with increased exposure to females, and is mite transmission affected by male mating rate?

The experiment provided no evidence the first mating in the two treatments differed, as expected from the identical mating histories of male ladybirds at this point. All ladybirds in both treatments (*N*=26 for 'frequent mating opportunity' and *N*=28 for 'standard mating opportunity') successfully acquired a mate on their first mating opportunity. The fraction of 'long' (<224 minutes) and 'short' (<224 minutes) copulations was similar in each treatment group (for 'frequent mating opportunity' treatment, 15 long copulations and 10 short with 1 not ascertained; for 'standard mating opportunity' treatment, 15 long copulations and 12 short with 1 not determined: Fisher exact test *P*=0.79). The probability of mite transmission was similar (for 'frequent mating opportunity' treatment, 18 copulations with mite transmission and 8 without; for 'standard mating opportunity' treatment, 22 copulations with mite transmission and 6 without: Fisher exact test *P*=0.54). Where mite transmission occurred, the intensity of infection was similar (for 'frequent mating opportunity' treatment, 4 copulations with low intensity of mite infection generated (1 to 5 larval mites) and 14 with high intensity (>5 larval mites); for 'standard mating opportunity' treatment, 7 copulations with low intensity of mite infection generated and 15 with high intensity: Fisher exact test *P*=0.72).

I then examined whether subsequent mating interactions were affected by the rate at which further interactions occurred ('frequent mating opportunity'= 10 hours later, 'standard mating opportunity'= 48 hours later). The probability of a male mating on any particular encounter with a female was not affected by the rate at which the females were provided to males. Males with 'frequent mating opportunity', provided with a female twice daily, procured the same number of matings as males with 'standard mating opportunity' provided with a female every two days (Figure 6.5).

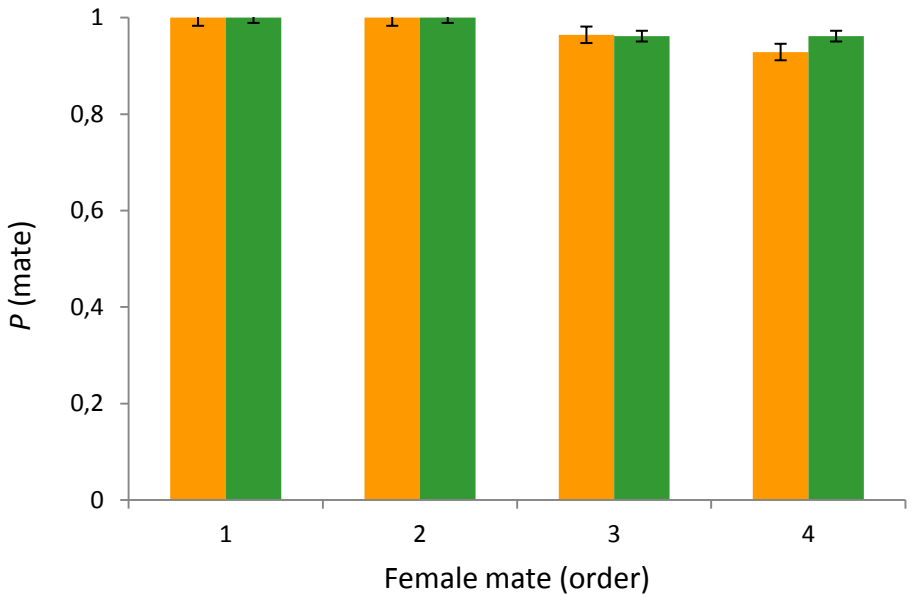


Figure 6.5: The probability that males mate with each of four females presented in turn, partitioned by rate of exposure of males to the female (green bars ● new female every 12 hours; orange bars ● new female every 48 hours). For each mating opportunity, *N*=26 for frequent *N*=28 for standard.

Statistical analysis did not reveal an effect of either treatment (how often males were offered females), sequence of mating (2nd, 3rd or 4th female), or an interaction between treatment and sequence, on the probability of mating occurring (Table 6.1). However, the rate at which females were provided with males did impact on the duration of copulation.

Table 6.1: Results of Binomial logit GLM on the response variable 'probability of male mating', examining contribution of presentation order of female and rate of presentation of females.

Factor	Df	Deviance	*P*
Exposure every 12 hours vs 48 hours	1	0.1383	0.71
Female sequence (order)	2	4.34	0.11
Interactions between treatment and female in order	2	0.1473	0.93

'Frequent' mating males (offered females twice per day) mated for shorter periods of time compared to males with 'standard mating opportunity' (offered a female every two days) (Figure 6.6). Splitting mating duration into 'long' and 'short' (see methods), the exposure in both 'frequent' and 'standard' treatment and sequence of mating opportunity explained variance in copulation duration, with no interaction term between these. Males with 'frequent mating opportunity' copulated for less time than males with 'standard mating opportunity', and subsequent copulations were more likely to be short in duration for both treatments (Table 6.2).

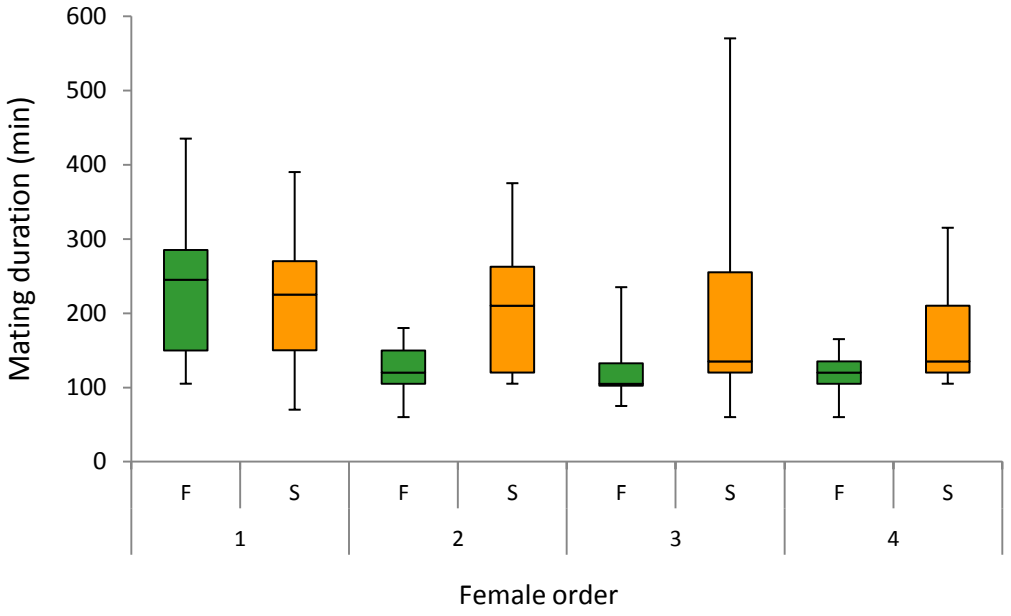


Figure 6.6: Boxplot of mating duration (minutes) for males presented to four females in order (1 = first, 4 = last), at two different exposure rates (green bars ● F = twice per day, orange bars ● S = once every two days).

Table 6.2: Results of Binomial logit GLM on the response variable 'duration of mating', examining contribution of presentation order of female and rate of presentation of females. Duration of mating is given as 'long'/'short' defined as above and below median duration on first exposure of a male to a female (224 minutes).

Factor	Df	Deviance associated with factor	*P*
Female sequence (order)	2	6.8	0.033
Rate of exposure of male	1	26.76	$2.3\ e^{-07}$
Rate of exposure x sequence	2	1.6	0.45

The development of mite infection, the second component of the mite transmission was then tested. The results showed that males with 'frequent mating opportunity' (offered females twice daily) were less likely to produce a mature mite infection on their female partners than males with 'standard mating opportunity' (offered males every second day) (Figure 6.7) (Table 6.3). There was no impact of order of mating in the male's mating sequence.

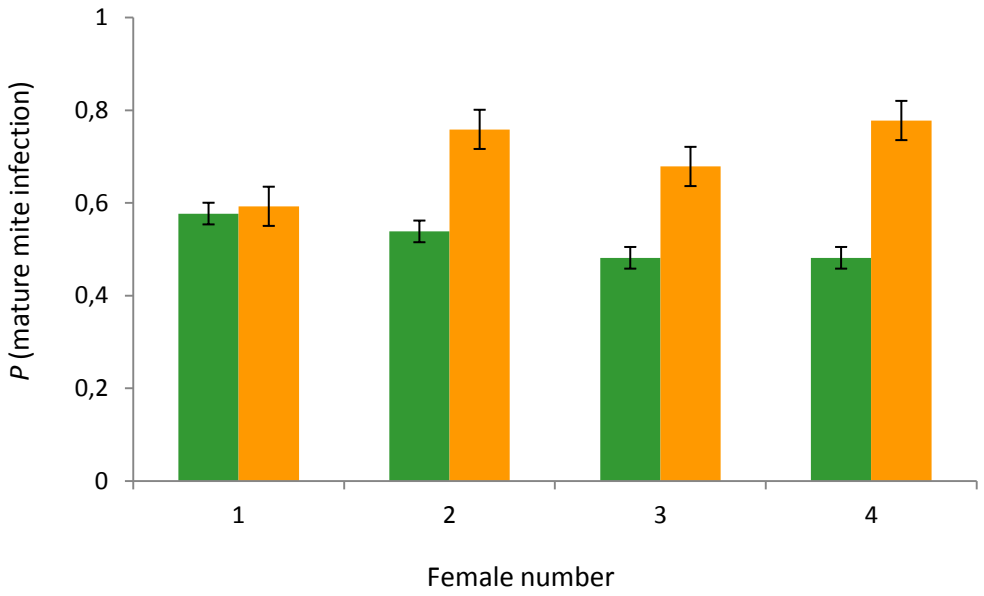


Figure 6.7: The probability of females developing a mature mite infection (as defined by adult mite presence 14 days following mating to a male) for males with different mating history. Prior mating history of the male partner is defined in terms of the position of the female in his order of his mating (1-4), and the rate at which he had been offered females (green bars ● two mating opportunities per day, orange bars ● one opportunity every 2 days). For 'Frequent mating opportunity', *N*=26 for first and second mating opportunity, *N*=25 third and fourth mating opportunity; For 'Standard mating opportunity', *N*=28 for first and second mating opportunity, *N*=27 for third, and *N*=26 for fourth.

Table 6.3: Results of Binomial logit GLM on the response variable 'presence of mature infection', examining contribution of presentation order of female and rate of presentation of females. Seq. L and Q refer to Linear and Quadratic impacts of order.

Factor		z value	*P*
'Frequent'/'Standard' male exposure history		2.804	0.005
Mating sequence	Seq. L Seq. Q	-0.538 0.23	0.590 0.818
Interactions between treatment and mating sequence	Seq. L Seq. Q	0.495 0.608	0.62 0.54

I further examined the components of the transmission process, examining initial transmission (presence of any larval mites on day 2, number of larval mites present on day 2) and development of infection (retention between day 2 and day 14). I began by testing the first part of the mite transmission, the probability of whether females initially acquired mite infection. Results and statistical analysis showed that there was no impact of male remating rate, order of mating, or the interaction between mating rate and order of mating on the possibilities of initial mite transmission (Figure 6.8 and Table 6.4).

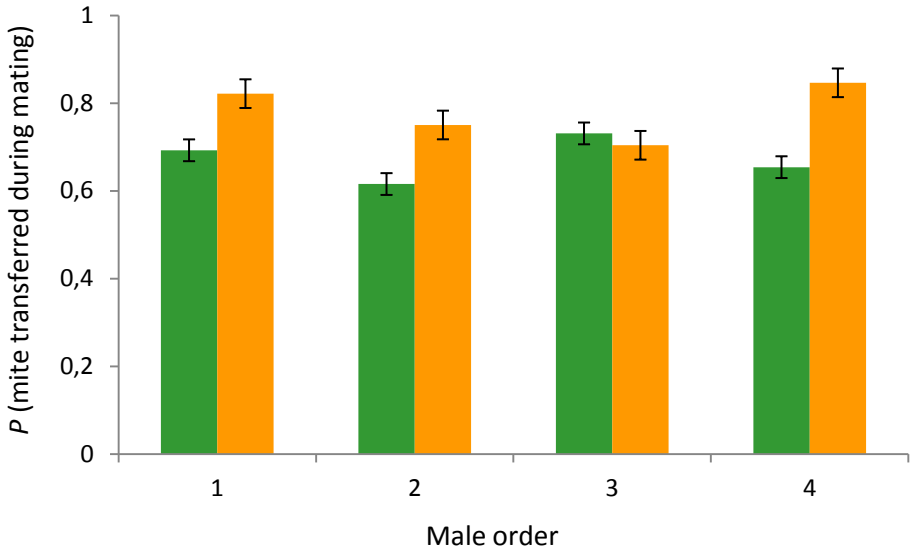


Figure 6.8: The probability that a male transfers larval mites to his female partner during mating, for different mating history of the male. Each mating is defined by position in the males order of his mating (1 being the first female, 4 the last), and the rate at which he is offered females (green bars ● two mating opportunities per day, orange bars ● one opportunity every 2 days). For 'Frequent treatment', *N*=26 for first and second mating opportunity, *N*=25 third and fourth mating opportunity; For 'Standard treatment', *N*=28 for first and second mating opportunity, *N*=27 for third, and *N*=26 for fourth.

Table 6.4: Results of Binomial logit GLMM on the response variable 'presence of larval mites', examining contribution of presentation order of female and rate of presentation of females. Seq. L and Q refer to Linear and Quadratic impacts of order within the mating sequence. No interaction term was present in the full model.

Factor		z value	*P*
'Frequent'/'Standard' mating opportunity		1.82	0.066
Mating sequence	Seq. L Seq. Q	0.540 -0.84	0.59 0.40
Mating rate x mating sequence	Seq. L Seq. Q	0.39 -1.67	0.69 0.09

However, remating rate did significantly affect the intensity of infection acquired (Figure 6.9). Analysing whether females acquired 'light' or 'heavy' infection (see methods) revealed that the males transmitted similar intensity of infection on the first mating in the sequence (as expected from their identical previous experience). But with the 'frequent mating opportunity' treatment, the intensity of infection declined particularly rapidly with the sequence order and those female partners were more likely to acquire a light mite infection (1-5 larval mites).

Statistical analysis revealed mating rate, accounted for variance in the initial intensity of infection (Table 6.5).

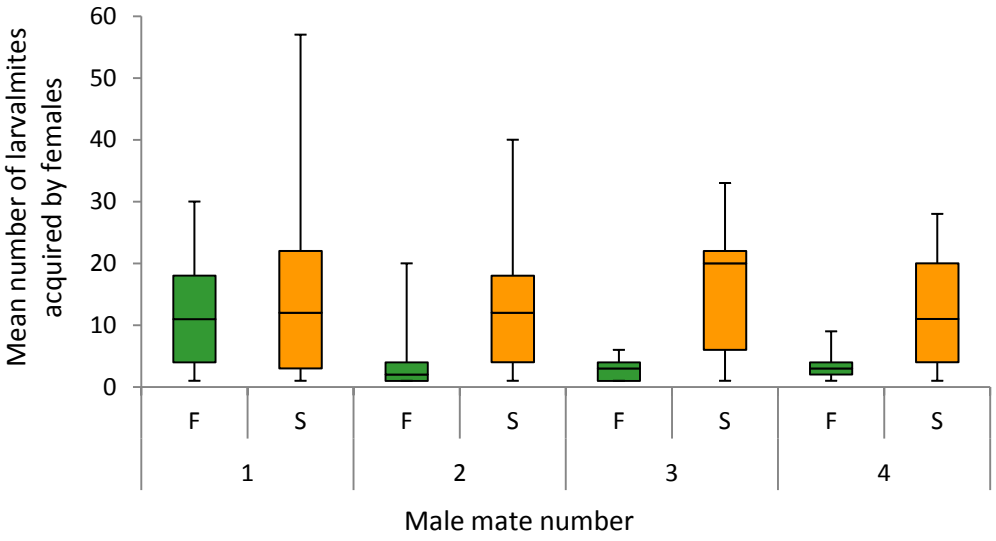


Figure 6.9: The mean number of larval mites acquired by females mated to a male with different mating history. Prior mating history of the male partner is defined in terms of the position of the female in his order of his mating (1 being the first female, 4 the last), and the rate at which he had been offered females (green bars ● two mating opportunities per day, orange bars ● one opportunity every 2 days).

Table 6.5: Results of Binomial logit GLMM on the response variable 'intensity of initial infection', classified as high vs low, examining contribution of rate of presentation of females and presentation order of female. Seq. L refers to Linear, Seq. Q to Quadratic impacts of order.

Factor		z value	*P*
'Frequent'/'Standard' male mating rate		5.34	$9.1\ e^{-08}$
Mating sequence	Seq. L Seq. Q	0.34 0.88	0.74 0.38
Mating rate x mating sequence	Seq. L Seq. Q	-0.83 -1.30	0.41 0.19

Furthermore it was also observed that low intensity infections were less likely to progress to mature infection: 30% of initially light infections did not progress to mature infection, compared to 4% of initially heavy infections (*N*=63 and *N*=56 respectively: 2x2 Fisher exact test *P*=0.002) (Table 6.6). There was a significant association between the categorical variables 'high intensity' and 'low intensity' on day 2 (high intensity defined as >5 larval mites and low intensity defined as 1-5 larvae) and 'presence'/'absence' of infection on day 14 (Chi-squared =25.88, df=1, *P*<0.000001).

Table 6.6: High or low initial intensity of mite infection at day 2nd and the presence of mite at day 14th.

	Infected day 14	Uninfected day 14
'Low' initial infection intensity	47	25
'High' initial infection intensity	82	3

6.3.2 Do females mate rapidly if they mated to a recently mated male?

When a recently mated female was offered a 'tester' male, the probability of interaction between the two was not affected by whether the female had previously mated to a 'recently mated' or a 'rested' male. 75/80 females previously mated to a 'rested' male were courted, compared to 71 of 80 females previously mated to a 'recently mated' male (GLM with male as random factor, *P*=0.281) (Table 6.7).

Table 6.7: GLMM model investigating impact of first male partner mating history on a female ladybird probability to interact with a second male, with male identity as a random effect.

Factor	Std. Dev.	Std. Error	z value	*P*
'Rested'/'Recently mated' first male partner	0.8947	0.6291	-1.077	0.281

In contrast, the previous mating history of their first male partner was associated with the female reaction to the approach by a second male. Females mated previously to a 'rested' male were more likely to reject mating opportunities than females that had previously mated to a 'recently mated' male. 23 rejected matings were observed in 71 interactions for females previously mated to a 'recently mated' male, compared to 63 rejected matings in 75 interactions for females previously mated to a 'rested' male, (GLMM model including male as a random effect, $P<0.0001$) (Table 6.8).

Table 6.8: GLMM model investigating impact of first male partner mating history on the probability of a female ladybird rejecting a second male with which she has interacted, with male identity as a random effect.

Factor	Std. Dev.	Std. Error	z value	*P*
'Rested'/'Recently mated' first male partner	1.052	0.4523	-6.422	$1.35e^{-10}$

6.4 Discussion

A wide variety of animals carry sexually transmitted infections, and the epidemiology of these infections will depend on the host mating system, as this has an impact on contact between host individuals through which transmission may occur (Knell and Webberley, 2004). Past considerations of STI epidemiology have emphasised the importance of mean and variance in mating rate, highlighting promiscuity and patterns of sexual selection in determining STI dynamics (Ashby and Gupta, 2013; Thrall *et al.*, 1997; Thrall *et al.* 2000).

In this study, laboratory experiments were used to examine the potential influences of population sex ratio bias on the dynamics of STIs. The model organism, *A. bipunctata*, both suffers from an STI, and experiences varying population sex ratios across its range, associated with presence of a male-killing symbiont (Ryder *et al.*, 2014). The experiments suggested first that male mating rate is plastic, and an increase in the availability of females, associated with strongly female-biased populations, is likely to be associated with an increase in the rate at which males mate. Further to this, when males mate more commonly, the female mating rate may also be higher as females are likely to remate more rapidly. As a balance to these features, which would enhance STI spread, female partners of mite infected males that remate commonly are about 30% less likely to develop mature mite infections, and rejected matings (probably more common in normal sex ratio populations) do have some (albeit much lower) potential for mite transfer.

These results highlight the likely impact of male-killer induced population sex ratio bias on STI epidemiology, but verification requires analysis in natural populations. In *Adalia*, males aggregate on plants where there are aphids and females are likely to be present. They then move within habitats and try to mate when they meet a female (Brakefield, 1984; Majerus, 1994). The data from experiments demonstrated that in the lab males are able to mate with females at four times the usual rate.

However it is still not well established whether ladybird males in the field are able to encounter females at the same rate as the laboratory ones. It is likely that a

strong female-biased sex ratio is associated with higher densities of females in the wild, thus encounter rates are likely to increase. However, experimental manipulation in the field will be required to establish this with precision.

Within this study one source of increased female mating rate has been identified. In the population with strongly female-biased sex ratio, males rapidly remate, and these males do not induce the inhibition of female to remate. In this situation if a female meets a male, she will mate. There are other factors which may also act to increase female mating rate where females are common. For instance, it was noted that males that remate rapidly copulate for less long compared to males that have not mated recently. In a mating system in which males search for females, this would result in males having more time searching for females, potentially also elevating female mating rate.

The results from this study suggest that shorter post-mating intervals of males between mating with female partners increases female mating rate in natural populations. If a male-killing bacterium is present in an *Adalia* population, the above hypothesis leads to prediction that female ladybirds in this population are more likely to acquire mite infection by virtue of also having an elevated mating rate. The presence of male-killing bacteria at high prevalence is expected to have a direct impact on male through mortality, females through male mortality, and then a second impact through their increased mite-induced sterility and morbidity (Ryder *et al.*, 2007).

Chapter 7: General Discussion

The primary aim of this thesis was to establish the factors driving the incidence and prevalence of mite and *Spiroplasma* infection in the two-spot ladybird *Adalia bipunctata*. The work presented demonstrates that there is a consistent pattern of mite incidence in Swedish populations of this ladybird, indicating a consistent cause of mite presence/absence. The hypothesis that variation in host phenology represents a major driver of mite incidence was presented. Data on host phenology indicated a lack of overlap between overwintered and new generation ladybirds in the north of Sweden, which would represent a 'hard stop' to mite persistence, as the mite would not be able to establish a new cohort in this geographical region.

I then examined whether the dynamics of *Spiroplasma* and mite might be linked. There were two main motivations for this:

a) There has been a recent appreciation that symbionts may be protective of their host against another parasite. Initially demonstrated in aphids (Oliver *et al.*, 2003), symbiont-mediated protection is now well established in a number of systems, and *Spiroplasma* themselves are known to induce protection against a variety of natural enemies. In other cases, symbiont presence is associated with profound increases in susceptibility to natural enemies. Any changes in susceptibility induced by a symbiont would link the dynamics of the two infections.

b) It is now recognised that the presence of one parasite or pathogen may alter the dynamics of another through changes in host demography. Parasite transmission commonly relies on contact between individuals – for a sexually transmitted parasite this contact is copulation. Any other parasite or pathogen that causes host mortality, or host sex ratio skew, may influence their contact rate, and from this affect the epidemiology of other parasites, independent of whether it affects individual susceptibility.

In this thesis, I both tested for protective effects and examined evidence that *Spiroplasma*-mediated changes in host demography would affect mite epidemiology. No evidence was found for the former. In contrast, elongation of

lifespan and sex ratio skew associated with *Spiroplasma* presence were both likely to alter mite epidemiology. Thus in this system, the direct impact of *Spiroplasma* on the mite through changes in individual susceptibility are low, but the indirect impacts, mediated through population level changes, are likely to be considerable.

I first summarise the findings from each chapter of this thesis. I then outline outstanding issues within the *Adalia*-mite and *Adalia*-*Spiroplasma* system before asking two general questions arising from the thesis:

a) Is it a general principle that STIs will become rare near the poles?

b) How common will it be that non-protective effects impact upon disease epidemiology?

7.1 Summary of findings

7.1.1 Chapter 2: The stability of Spiroplasma and mite presence in Swedish ladybird populations

In this Chapter I presented data indicating that the distribution of the mite in Sweden parallels that observed in 2000-2002, being absent north of 61˚N and in Nässjö (the only southerly site), but found commonly in other southern ladybird population. This constancy indicates that there is likely to be a consistent factor driving incidence of the mite over time, in contrast to either metapopulation or co-evolutionary dynamics.

The distribution of male-killing bacteria observed in this study (2011-2013) was found to be broadly similar to that observed in surveys conducted 10 years ago (2000-2002). *Spiroplasma* remains the most dominant symbiont in all ladybird collections. Its prevalence remained high in the south of Sweden, whereas it was rare or absent in northern populations. However, there is some evidence that the *Spiroplasma* range has slightly expanded northwards. *Rickettsia* remained at low prevalence throughout Sweden. Finally, there is the appearance of *Wolbachia*-infection. *Wolbachia* was not recorded in the 2000-2002 survey, but was found at

low prevalence in the 2011-2013 samples. Thus, this study provides data which shows the coexistence of multiple male-killing bacteria in some ladybird populations, as found previously in the population from Moscow (Majerus *et al.,* 2000).

Taking the observations on mites and symbionts together, this chapter establishes that male-killing bacteria are common in many locations where the mite exists. It thus indicates the need to ascertain the interaction between symbiont and mite, both at individual and population levels.

7.1.2 Chapter 3: Host phenology limits the incidence of a sexually transmitted infection

There is little empirical work on the determinants of STI incidence in natural populations. In this Chapter I explored the possible cause for mite absence from the northern ladybird populations and from Nässjö (the only southern site where mites were absent). Previous studies (Hurst *et al.*, 1995; Knell and Webberley, 2004; Webberley *et al.*, 2006a,b) suggested that the ability of the mite to transfer between different generations of *Adalia bipunctata* is the principle determinant of mite persistence. This transfer depends on adults from both overwintered and newly emerged generations mating. Whether this occurs depends on host phenology, which depends in turn on temperature and food resources, and the length of the ladybird reproductive season. I observed that moving north in Sweden is associated with a delay in reproduction by overwintered adults to later in the year, and a delay in the emergence of the next cohort. As a result of this, overwintered ladybirds in the north die before a new cohort of adults emerges. This leads to the conclusion that ladybird phenology does not allow for contact between generations. Therefore the mite cannot be transmitted, maintained and persisted in the north of Sweden.

7.1.3 Chapter 4: No evidence that the presence of sexually transmitted infection selects for reduced mating rate in the two-spot ladybird, Adalia bipunctata

Theory has predicted that the presence of an STI should be selected for a reduction in mating rate of female hosts (Kokko *et al.*, 2002; Boots and Knell, 2002). The predictions of this theory, that populations with an STI should be selected for increased tendency to reject matings compared to uninfected populations, have never been tested. In this chapter the efficiency of ladybird rejection behaviours in preventing STI was established. However, contrary to the theory above, rejection behaviours were not more commonly observed in the populations where mite is present (in this study Stockholm) compared to populations where mite is absent (in this study Nässjö). Combined with previous work showing lack of discrimination of mating partners with respect to STI infection status (Webberley *et al.*, 2002), the results indicate that STIs do not strongly drive the evolution of mating behaviours, contrary to theory.

7.1.4 Chapter 5: Spiroplasma do not alter STI epidemiology through protective or phenological effects

Recent studies have established that heritable bacteria can protect their host against natural enemy attack (Oliver *et al.*, 2003; Hedges *et al.*, 2008). This chapter found no evidence for *Spiroplasma* symbiont-mediated protection of ladybirds against mite attack. There was no evidence that *Spiroplasma* prevented acquisition or development of the mite infection, and did not prevent the infertility impact. Thus, I conclude that this symbiont is not protective against the mite.

A second potential individual impact of *Spiroplasma* infection is on host longevity. Elnagdy *et al.* (2013) presented data that indicates that *Spiroplasma*-infected ladybirds lived much longer than ladybirds free from symbiont infection. A longevity impact would aid mite persistence through increasing overlap between cohorts. In this chapter, I examined whether this result of increased longevity was found in beetles collected from the field following overwintering, and whether it was retained or altered by the presence of mites.

In the absence of the mite, female ladybirds infected with *Spiroplasma* lived longer than those which didn't carry the *Spiroplasma* infection. This difference was not apparent when the mite was present, such that longevity is high with either mite or *Spiroplasma* present. Though the *Spiroplasma* showed a positive effect for ladybird longevity in my study, the magnitude of effect (c. 20 days difference in LT50) was much smaller than the 120 day difference previously observed (Elgnady *et al.*, 2013). Interestingly, the mite itself did not negatively impact on ladybird survival, and indeed mite-infected ladybirds were more long lived (compared to mite free beetles) when *Spiroplasma* was absent. Together, these data indicate that *Spiroplasma* may aid mite persistence, by increasing the period of overlap between mite infected ladybirds from the overwintered cohort and individuals from the new cohort. Unexpectedly, the mite may similarly aid its persistence through reducing mortality of its host.

7.1.5 Chapter 6: Assessing the impact of symbiont induced sex ratio bias on the dynamics of sexually transmitted infections in the two-spot ladybird Adalia bipunctata

Recent studies have indicated the importance of viewing parasites and pathogens in a community context, as the actions of one natural enemy on (e.g.) population size affects the transmission of others. In the population where a male-killing bacterium is present, sex ratio bias is observed. For instance, the population sex ratio in Stockholm reaches 3-4 females per male. In female-biased populations mating rate is higher for males than females, meaning that males are therefore more exposed to mite infection. Previous study has demonstrated that male ladybirds become infected with mites approximately two weeks earlier than females in the population where the male-killing bacteria are common (Ryder *et al.*, 2014).

In this Chapter I investigated the impact of sex ratio bias in the ladybird population on the STI epidemiology in more detail. In particular, I used laboratory study to assess if sex ratio bias was likely to be associated with higher absolute mating rate: that is to say if increased availability of female partners produces an increase in mating rate. The experiments showed that male mating rate is plastic, and males

mate readily with every female they meet. However, it was also observed that if a mite-infected male ladybird mates very often then he is less likely to transmit sufficient larval mites, to produce a mature infection, on his partner. This reduces the speed of the epidemic on female beetles. Finally, it was observed that female mating rate may increase when males are rare, because males that mate commonly do not inhibit remating by their female partner. The impact of sex ratio biases on mite dynamics is thus complex, and demonstrates the importance of understanding the behaviour underpinning mating decisions, and recognising that 'per contact' parasite transmission rates may decline with increasing transmission opportunities.

7.2 Outstanding issues within the *Adalia*-mite and *Adalia*-*Spiroplasma* system

7.2.1 If Spiroplasma influences the mite epidemiology, what is driving Spiroplasma incidence/prevalence?

This thesis has made progress in uncovering the determinants of mite incidence, and has revealed how the mite may interact with *Spiroplasma* through population level impacts of sex ratio bias. However, the determinants of *Spiroplasma* prevalence variation remain obscure. There are two aspects of *Spiroplasma* prevalence to explain. First, why is prevalence higher in southern Sweden than nearby continental European populations (e.g Poland, Denmark)? Whilst *Spiroplasma* prevalence in Malmö is 35.8% (*N*=123), this symbiont was not recorded in Ribe, Denmark (*N*= 43) (Hurst *et al.*, 1999a) and occurs at 3.6% prevalence in Toruń, Poland (Ryder *et al.*, 2014). Second, why does the *Spiroplasma* prevalence decline in the far north?

Neither of these patterns is easily understood. High prevalence would be expected to follow from there being a high benefit to male-killing, high transmission efficiency, low direct costs or presence of a direct benefit to infection. Possible local direct benefits could derive from local natural enemies. This thesis has however excluded the mite as a strong driver of *Spiroplasma* presence, both from direct examination of the interaction, and lack of concordance between *Spiroplasma* and mite incidence (Nässjö has high *Spiroplasma* incidence without mite infection). The

continued presence of *Spiroplasma* at high prevalence levels suggests that the cause of variation is not coevolutionary, as parasite-host arms race cycles would be expected to show variation over time. An unrecognised ecological factor affecting either the benefit of male-killing, the efficiency of transmission, or the cost of infection is thus likely. One hypothesis worth exploring is whether high summer temperatures impact on *Spiroplasma* transmission, as this would partly explain the rareness of *Spiroplasma* in continental Europe.

The causes of the northern boundary to high prevalence levels of *Spiroplasma* infection are also enigmatic. The northern parts of Sweden are characterised by longer winters and a consequentially compressed reproductive season. Majerus and Majerus (2012) suggest that the advantage of male-killing declines in these conditions, as a compressed summer is associated with aphids being abundant when ladybirds are reproducing. Rich food resources reduce the benefit to male-killing by a symbiont because larval females have enough food and gain little from consuming their siblings. It is also notable that the main host plant for ladybirds changes in northern populations. Whilst ladybird reproduction in southern cities is largely on lime trees, these are absent in the north, where birch is instead the major habitat. This creates a direct change in diet and resource availability, notwithstanding any seasonal impacts on aphid phenology.

An alternative explanation for northern rarity of *Spiroplasma* is that there might be increased *Spiroplasma*–induced costs during diapause in the presence of very low temperatures. Tinsley (2003) examined the impact of prolonged overwintering at 0.15˚C on ladybird survival and *Spiroplasma* transmission. *Spiroplasma* infection did not affect overwinter survival, and did not have decreased vertical transmission after overwintering.

Further work should investigate whether temperatures below zero represent an additional physiological problem. Sub-zero temperatures present the risk of ice crystal formation, creating damage to cells and ultimately insect mortality. Invertebrates survive winter by use of cryoprotectants, which may be antifreeze proteins or high levels of haemolymph trehalose (Convey, 1996; Storey and Storey,

1996). It is thus important to establish how *Spiroplasma*-infected beetles react to extreme low temperatures that occur in the north. Work on *Ixodes* ticks has demonstrated that *Anaplasma* symbiont infection is associated with increased expression of antifreeze proteins in *Ixodes* ticks, and increased cold tolerance (Neelakanta *et al.*, 2010). *Spiroplasma*-infected cicadellid bugs likewise show improved overwinter survival (Summers *et al.*, 2004). Thus, the impact of very low temperatures on *Spiroplasma*-infected/uninfected female ladybirds should be investigated.

7.2.2 Why do ladybirds appear to have no mechanism for preventing STI infection or progression?

Parasites are regarded as strong evolutionary forces, driving selection to avoid infection and to prevent disease-induced morbidity/mortality. However, despite mite infection being very common, there is no evidence for any evolutionary response in the host. There is no evidence for avoidance through reduced mating rate (this thesis), no evidence for avoidance of infected partners when they are encountered (Webberley *et al.*, 2002). In Chapter 3, it was further noted that ladybirds from Stockholm (mite present) showed no difference in their ability to prevent either acquisition or progression of mite infection, compared to ladybirds from populations where the mite was not present.

Why is there no mate choice or impact on mating rate? It has been widely conjectured that mate choice should include contagion avoidance and that STIs should select on mating rate. Mate choice in ladybirds relies on a female rejecting mating by a male which does not court, but instead climbs onto the female. Rejection behaviours include raising the abdomen within the elytra, kicking the male and rolling over (Webberley *et al.*, 2002). In this study, rejection behaviours were shown to be protective against infection, but were not fully protective. It is possible that prolonged rejection is not protective, as greater contact time between partners permits mite transfer. Further, rejection is probably energetically costly to the female. Thus, it may be that male persistence in attempting mating is the core reason underlying lack of selection on female mating behaviour. Whilst it is

beneficial to reject if rejection works quickly, it is less beneficial and may be costly where it involves a prolonged encounter.

The lack of immune defence against the mite is also mysterious given the frequency with which mite infection occurs and its profound cost, the sterility of the female host. One possibility is that the May/June epidemics of the mite coincide with a period where short-term reproduction is very important. Because there are high levels of egg, larval and pupal mortality associated with intra-guild predation and cannibalism, ladybird fitness probably corresponds strongly to output during the early phase of reproduction. Serious mite impacts that occur during late reproduction may not be very important in terms of fitness due to cannibalism by previously hatched larger larvae/lack of preparation of any emerging ladybirds for winter. This would mean that there is only weak selection to avoid infection after winter.

Above prediction that STIs select for lower mating rate applies mostly to female hosts. We know that there are smaller benefits for females with each additional mating and higher cost of infection (sterility). However, it is still not well understood how the STIs influences male mating behaviours. For males each mating opportunity provides significant fitness benefits and the STI cost for males is weak (males do not become sterile). As a result, selection on males is expected to act in a different way (Ryder *et al.*, 2007). Possibly, when a large proportion of female hosts are infected with mites and consequently infertile, male mating behaviour might be altered. Males might evolve to shift their life history strategy and invest in more frequent early-season copulation attempts. Early in the season only 10% of ladybirds carry the mite, therefore males have more opportunities for successful mating and successful spread of the genes. If this happened, this might lead to convenience polyandry selection on females.

7.2.3 How does Spiroplasma increase longevity? Is this general?

Combined data from Elnagdy *et al.* (2013) and from this study have provided evidence, however not so strong, that *Spiroplasma* bacteria increase longevity of their ladybird host. Future work should revalue the *Spiroplasma* impact on host

longevity. To this date, it is unknown how *Spiroplasma* increases the longevity. One possible interaction is with host metabolism. It is known from many species that caloric restriction is associated with increased longevity. Eighty years ago, McCay *et al.* demonstrated that caloric restriction of rodents has a positive effect on maximal life span (McCay *et al.*, 1935). Since this finding numerous studies have shown the same results in many other animal species. For instance, a recent study on rhesus monkeys showed that caloric restriction reduces age-related mortality and also reduces the impact of other factors causing mortality (Colman *et al.*, 2014).

Physiological changes that occur when an animal undergoes caloric restriction include: reduced body temperature and weight, reduced glucose and insulin levels and also lower metabolic rate. However, it is still unknown which of these changes are essential for prolonged life span (Lakowski and Hekimi, 1998). My observations of ladybirds suggest that calorie restricted beetles live longer. Ladybirds maintained on an artificial diet (sugar and water) lived longer than those maintained with aphids. One possibility is that *Spiroplasma*, in using host energy for replication and maintenance, acts as an internal modulator of calorie availability, producing the effect on longevity observed.

A second possibility is that symbionts affect the stress tolerance of their host. Whilst not known for insects, this is established for fungal symbionts of plants. Rodriguez *et al.* (2008) showed that fungal symbionts produce stress tolerance for their plant host depending on the habitat, and this stress tolerance allows plant to live longer. It may be that this occurs because the symbiont induces stress responses in its host, and thus primes it for deteriorating environmental circumstances.

7.3 General perspective arising from this thesis

7.3.1 Is it a general principle that STIs will become rare near the poles?

Collections of two-spot ladybirds within Europe over the last 10 years have noted that the sexually transmitted parasitic mite is widely present across European

ladybird populations but absent from populations near the north-west coast and from northern Scandinavian (Webberley *et al.*, 2006b). This thesis shows that contact between ladybirds from overwintered and new generation cohorts is one important requirement for mite presence and persistence in the ladybird population.

The extension of this thinking suggests that STI incidence in insects in general will decline towards the poles. In *Adalia*, northern parts of Europe were characterized by a compressed reproductive season where cohort overlap is less likely because old ladybirds die before new ones hatch from pupae. In other species, there may be a more pronounced effect, associated with obligate diapause in temperate/polar regions. In the seven-spot ladybird *Coccinella septempunctata*, for instance, there is genetic variation in diapause requirement, with ladybirds from northern climes not entering into sexual activity until emergence from overwintering (Phoofolo and Obrycki, 2000; Hodek *et al.*, 2012). This type of 'hard requirement' for individuals from northern climes to undergo diapause before reproduction is seen in other species (Saunders, 1970). A prediction of my thesis is that the mite *C. macfarlenei* should be absent from populations where obligate diapause is common. The incidence of sexually transmitted mite would correspond to the geographical region where there is no diapause requirement.

In addition to this, the required sexual contact between generations for STI persistence might be correlated with voltinism. Univoltine reproduction (one main generation per year), occurs in areas with more mild spring and summer temperatures, and it is likely to be associated with the presence of generation gap, for instance in the north of Sweden and in Britain (Hurst *et al.*, 1995; Webberley *et al.*, 2006b). In contrast, multivoltine reproduction (more than one main generation per year) exists in areas with high spring and summer temperatures and where generations overlap, for instance in the south of Sweden, in Central Europe and in Moscow (Russia) (Webberley *et al.*, 2006b). Therefore, overwintered ladybirds from the southern populations that eclose later in the year, might be younger and survive longer in the following reproductive season.

7.3.2 How commonly do non-protective effects impact upon disease epidemiology?

There has been a large emphasis on the protective effects of symbionts, mediated by the revelation that diverse heritable microbes make their host resistant to infection by diverse natural enemies in a range of insect host species. In this thesis, no protective effects were observed, but a strong case was made that the symbiont would affect the epidemiology of the STI through its indirect effects. In *Adalia, Spiroplasma* induced male-killing alters the mating system, and thus STI dynamics.

How widely in nature will such indirect effects occur? Male-killing bacteria commonly produce highly biased population sex ratios, with male hosts becoming rare. There is much literature on vertebrates demonstrating that male and female hosts have different intrinsic susceptibility to parasites, with testosterone associated with immunosuppression and male-biased parasitic infection (Grossman, 1985; Moore and Wilson, 2002). Whether such effects exist in invertebrates, where there are no 'androgens' of this type and immune systems do not have an acquired component, is more contentious. Literature review suggests no general trend for male-biased parasitism in insects (Sheridan *et al.*, 2000). However, Sheridan *et al.*, (2000) highlighted nine of 24 case studies in which sex-biased parasitism was observed, the difference being that this was a mix of male- and female-biased parasitism. Interactions such as these would be modulated by sex ratio distorting symbiont presence.

Sex ratio distortion is, additionally, just one phenotype of symbionts that may impact on disease dynamics. Moreover there are also endosymbiont affect host dispersal behaviours. Goodacre *et al.* (2009) has demonstrated that the microbial agents influence long-distance dispersal of ballooning female spiders *Erigone atra*. In this species, *Rickettsia*-infected individuals were less likely to disperse. Dispersal dynamics of hosts and parasites are important contributors to disease dynamics at a landscape scale (Goodacre *et al.*, 2009), and host and parasite dispersal might have an effect on shaping the evolution of parasite virulence (Lion *et al.*, 2006). Symbiont impacts on dispersal are thus very likely to alter parasite epidemiology.

Finally, symbionts can affect host longevity, as demonstrated in this study and elsewhere. For parasites and pathogens, there is a latent period during which the host is infected but not infectious. High host survival is required for high rates of onward transmission. Studies of symbiont impact on longevity are rather few in number, compared to studies of impacts on fecundity, as the experiments are more onerous. However, effects may be common. Fukatsu *et al.* (2001) has showed that the *Spiroplasma* symbiont negatively affect the growth, reproduction and longevity of its host aphid *Acyrthosiphon pisum.* Madden *et al.* (1984) has observed that Corn Stunt Spiroplasma (CSS) reduced the probability of survival in leafhopper *Dalbulus elimatus* and *D. gelbus*. Each of these interactions would reduce onward transmission opportunities for other pathogens.

7.3.3 Wolbachia emergence and the presence of male-killing bacteria co-infection in the two-spot ladybirds populations – is it real?

Aside the northward movement in *Spiroplasma* prevalence, this survey revealed the emergence of *Wolbachia* symbiont infections in a small number of beetles. The source of this symbiont infection remains unclear. Two possibilities could be considered in order to explain the difference in the *Wolbachia* presence between previous survey and this study. First, it could be possible that the techniques used around 10 years ago were not good enough to detect *Wolbachia* presence in ladybirds. Second, it is possible that there was error in data of this study. Contamination of some samples during PCR assays could lead to the appearance of false bands in gels. Although this symbiont is rare and might not be an important driver of ladybird population sex ratio, it is important to understand if this appearance was real. In order to validate which of above hypotheses is true, patterns of mitochondrial DNA (mtDNA) polymorphism could be used to understand the selection pressures acting on the cytoplasmic genome. Both bacteria and mtDNA are maternally transmitted (Jiggins and Tinsley, 2005). Maternally inherited microorganisms can influence the mtDNA pattern of variation in hosts. This influence is driven by selection among symbionts and can cause the frequency of mitochondrial variants in the population to eventually increase or decrease (Ilinsky, 2013). Previous study has shown that male-killing bacteria and mt

DNA are in linkage disequilibrium within *Adalia bipunctata* populations (van der Schulenburg *et al.*, 2002). It has been also revealed that male-killing bacteria *Rickettsia* are associated with high levels of mitochondrial polymorphism. Therefore, this method seems to be sufficient to test the association between mtDNA haplotypes and *Wolbachia* genotypes.

Further, PCR assays also suggested the presence of co-infected ladybirds – ones with *Spiroplasma* and either *Rickettsia* or *Wolbachia*. This interesting aspect has not been discussed further because the sporadic nature of these co-infections made it hard to assess if they were simply a product of false positive PCR assays or occasional contaminants. However, if we would like to understand the symbiont interactions better, it is important to assess if these co-infections were real. The presence of co-infection can be established through sequencing samples and comparing these DNA sequencing results against the previously acquired sequences from ladybird symbionts. Previous studies have tried to explain the coexistence of these four male-killing bacteria within a single population, this coexistence is still not well understood. To address the dichotomy between observation of the presence of multiple symbionts in one population and predictions made by a model of evolutionary male-killing bacteria dynamics, in the future studies should focus on few aspects. First, researchers should focus on monitoring fluctuations in the prevalence of these endosymbionts over time. Secondly, studies should establish whether *Adalia bipunctata* ladybirds are equally susceptible to each of these male-killing bacteria. Finally, future work should also ascertain possibilities of niche separation in the utilisation of host by symbionts (Majerus *et al.*, 2000).

In summary, this study has revealed how symbionts may influence disease epidemiology mediated through sex ratio bias. Symbionts are commonly cryptic in natural populations, and investigating the impact of symbionts on host individuals requires considerable work. Future work should establish not just the impact of symbionts on the host individual, but also their effect on populations and the community of other parasites that utilize the host.

Appendix

Tables/Figures are labelled by the chapter from which they apply. E.g. Table A2.1 from Chapter 2.

Table: A2.1 Presence/absence of mite *C. hippodamiae* in various Scandinavian populations of *Adalia bipunctata* in years 2000-2002 and in 2011, 2012 and 2013.

Location	Latitude Longitude	Altitude	Range of average monthly temperature	2000, 2001 and 2002 (Tinsley, 2003; Webberley *et al.,* 2006b)			2011			2012			2013		
				N	Mite present?	Mite %	*N*	Mite present?	Mite %	*N*	Mite present?	Mite %	*N*	Mite present?	Mite %
Narvik (Norway)	68˚25’ N 17˚33’ E	17 m (56 ft)	-8˚C to 17˚C	84	0	0	-	-	-	-	-	-	-	-	-
Vilhelmina (Sweden)	64˚37’ N 16˚39’ E	347 m (1147 ft)	-23˚C to 18˚C	43	0	0	28	0	0	-	-	-	-	-	-
Östersund (Sweden)	63˚11’ N 14˚40’ E	312 m (1,024 ft)	-10˚C to 19˚C	494	0	0	51	0	0	99	0	0	9	0	0
Ljusdal (Sweden)	61˚50’ N 16˚05’ E	145 m (476 ft)	-10˚C to 21˚C	1215	0	0	189	+	0.53	92	0	0	-	-	-
LATITUDE 61˚															
Gävle (Sweden)	60˚40’N 17˚10’ E	68 m (224 ft)	-7˚C to 21˚C	41	+	12.2	199	+	40.2	54	+	5.27	-	-	-
Helsinki (Finland)	60˚10’ N 24˚56’ E	51 m (167 ft)	-6˚C to 17˚C	-	-	-	40	0	0	-	-	-	-	-	-
Stockholm (Sweden)	59˚19’ N 18˚4’ E	52 m (171 ft)	-5˚C to 23˚C	227	+	89.9	3041	+	42.5	146	+	23.8	567	+	7.4
Tartu (Estonia)	58˚38’ N 26˚72’ E	67 m (219 ft)	-11˚C to 24˚C	-	-	-	-	-	-	44	+	22.7	-	-	-
Nässjö (Sweden)	57˚39’ N 14˚41’ E	375 m (1230 ft)	-7˚C to 21˚C	69	0	0	108	0	0	76	0	0	-	-	-
Malmö (Sweden)	55˚35’ N 13˚02’ E	73 m (240 ft)	-3˚C to 22˚C	276	+	6.2	200	+	54.5	-		-	-	-	-

Table A2.2: Prevalence of different male-killing bacteria *Spiroplasma* (S), *Wolbachia* (W) and *Rickettsia* (R) in females from various populations of *Adalia bipunctata* in years 2000-2002 and in 2011, 2012 and 2013.

Location	Latitude Longitude	Altitude	Average temperature	Male-killing bacteria prevalence (according to Tinsley, 2003)								Male-killing bacteria prevalence (according to this study)											
				2000				2001/2002				2011				2012				2013			
				N	S%	W%	R%	*N*	S%	W%	R%	*N*	S%	W%	R%	*N*	S%	W%	R%	*N*	S%	W%	R%
Narvik (Norway)	68˚25’ N 17˚33’ E	17 m (56 ft)	-8˚C to 17˚C	245	0	0	1.22	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Vilhelmina (Sweden)	64˚37’ N 16˚39’ E	347 m (1147 ft)	-23˚C to 18˚C	-	-	-	-	99	9.1	0	15.2	19	10.5	0	0	-	-	-	-	-	-	-	-
Östersund (Sweden)	63˚11’ N 14˚40’ E	312 m (1,024 ft)	-10˚C to 19˚C	56	1.8	0	3.6	65	3.1	0	9.2	22	0	0	4.5	59	3.4	0	3.4	6	0	0	33.3
Ljusdal (Sweden)	61˚50’ N 16˚05’ E	145 m (476 ft)	-10˚C to 21˚C	-	-	-	-	75	10.7	0	25.3	181	26.5	2.2	14.4	59	79.7	1.7	8.5	-	-	-	-
LATITUDE 61˚																							
Gävle (Sweden)	60˚40’ N 17˚10’ E	68 m (224 ft)	-7˚C to 21˚C	-	-	-	-	141	51.8	0	5.0	217	42.4	4.6	3.7	47	61.7	2.1	0	-	-	-	-
Helsinki (Finland)	60˚10’ N 24˚56’ E	51 m (167 ft)	-6˚C to 17˚C	-	-	-	-	-	-	-	-	40	82.5	2.5	0	-	-	-	-	-	-	-	-
Stockholm (Sweden)	59˚19’ N 18˚4’ E	52 m (171 ft)	-5˚C to 23˚C	29	34.5	0	3.4	128	38.3	0	0.8	585	46.3	5.1	2.2	551	45.0	2.9	7.3	412	41.0	4.1	9.0
Tartu (Estonia)	58˚38’ N 26˚72’ E	67 m (219 ft)	-11˚C to 24˚C	-	-	-	-	-	-	-	-	-	-	-	-	44	47.7	9.1	4.5	-	-	-	-
Nässjö (Sweden)	57˚39’ N 14˚41’ E	375 m (1230 ft)	-7˚C to 21˚C	-	-	-	-	93	35.5	0	6.5	159	47.8	5.7	6.9	50	50.0	8.0	10.0	-	-	-	-
Malmö (Sweden)	55˚35’ N 13˚02’ E	73 m (240 ft)	-3˚C to 22˚C	46	34.8	0	0	139	32.4	0	0	123	35.8	0.8	3.3	-	-	-	-	-	-	-	-

Table A2.3: Details of *Adalia bipunctata* collections made at Scandinavian sites in 2000 (according to Tinsley, 2003). Sites are listed in order of declining latitude, with northerly samples on the top and southerly samples at the end of the table.

	Site	Date	Females	Males	Host plant
Spring 2000	Östersund	30/5-1/6	95	86	*Sorbus aucuparia, Acer sp.*
	Stockholm	29-30/5	169	94	*Tilia sp., Acer sp., Fagus sp.*
	Malmö	25-28/5	131	31	*Tilia sp., Ulmus sp.*
Autumn 2000	Narvik (Norway)	14-15/9	304	307	*Betula sp.*
	Malmö	11/9	119	68	*Tilia sp., Ulmus sp., Salix sp.*

Table A2.4 Details of *Adalia bipunctata* collections made at Scandinavian sites in 2001 (according to Tinsley, 2003). Populations are listed in order of declining latitude, with northerly samples on the top and southerly samples at the end of the table.

	Site	Date	Females	Males	Host plant
Spring 2001	Vilhelmina	25/6	44	43	*Betula sp.*
	Östersund	23-24/6 +26/6	93	92	*Betula sp., Prunus padus*
	Ljusdal	22-23/6	77	60	*Tilia sp., Rosa sp.*
	Gävle	21-22/6	146	50	*Tilia sp.*
	Stockholm	21/6 +27/6	137	42	*Tilia sp.*
	Nässjö	20/6	115	69	*Tilia sp., Rosa sp.*
	Malmö	19/6 +28-29/6	156	57	*Tilia sp., Ulmus sp.*
Summer 2001	Vilhelmina	26/7	118	99	*Betula sp.*
	Östersund (Pupal)	25/7 +27/7	140	123	*Betula sp.*
	Stockholm	24/7	128	70	*Tilia sp.*
Autumn 2001	Östersund	19/9	140	105	*Betula sp.*
	Stockholm	18/9	167	58	*Tilia sp.*
	Malmö	20/9	112	59	*Tilia sp., Ulmus sp.*

Table A2.5 Details of *Adalia bipunctata* collections made at Scandinavian sites in 2002 (according to Tinsley, 2003). Populations are listed in order of declining latitude, with northerly samples on the top and southerly samples at the end of the table.

	Site	Date	Females	Males	Host plant
Spring 2002	Östersund	8-9/6	261	389	*Betula sp.*
	Stockholm	7/6	231	127	*Tilia sp.*
	Malmö	6/6	185	91	*Tilia sp., Ulmus sp.*
Autumn 2002	Östersund	11/9	235	259	*Betula sp.*
	Ljusdal	12-15/9	478	327	*Sorbus aucuparia, Betula sp., Tilia sp.*
	Stockholm	10/9	157	70	*Tilia sp.*
	Malmö	9/9	249	170	*Tilia sp., Ulmus sp.*

Table A3.1: Phenological observation taken in 2000 at various points in Scandinavia (Tinsley, 2003).

Site	Latitude Longitude	Altitude	Average temperature	Date	Phenology observations
Malmö	55˚35'N 13˚02'E	73 m (240 ft)	-3˚C - 22˚C	25-28/05	Eggs, larvae of all instars and some pupae present. Season well progressed.
Stockholm	59˚19'N 18˚4'E	52 m (171 ft)	-5˚C - 23˚C	29-30/05	Larvae and eggs very rare, no pupae. Early season.
Östersund	63˚11'N 14˚40'E	312 m (1,024ft)	-10˚C - 19˚C	30/05-1/06	Overwintered adults that had recently come out from diapause, no eggs.
Malmö	55˚35'N 13˚02'E	73 m (240 ft)	-3˚C - 22˚C	11/09	Population almost exclusively adults, larvae and pupae extremely rare.
Narvik (Norway)	68˚25'N 17˚33'E	17 m (56 ft)	-8˚C - 17˚C	14-15/09	Mixed young adult and pupal sample. Pupae were emerging at time of collection. Late season.

Table A3.2: Phenological observation taken in 2001 at various points in Scandinavia (Tinsley, 2003).

Site	Latitude Longitude	Altitude	Average temperature	Date	Phenology observations
Malmö	55˚35'N 13˚02'E	73 m (240 ft)	-3˚C - 22˚C	19/06 +28-29/06	All developmental stages present. Eggs abundant.
Nässjö	57˚39'N 14˚41'E	375 m (1230 ft)	-7˚C - 21˚C	20/06	All developmental stages present.
Stockholm	59˚19'N 18˚4'E	52 m (171 ft)	-5˚C - 23˚C	21/06 +27/06	Fourth instar larvae and pupae common. Many newly emerged adults.
Gävle	60˚40'N 17˚10'E	68 m (224 ft)	-7˚C - 21˚C	21-22/06	Newly emerged adults.
Ljusdal	61˚50'N 16˚05'E	145 m (476 ft)	-10˚C - 21˚C	22-23/06	Adults and pupae rare. Many fourth instar larvae.
Östersund	63˚11'N 14˚40'E	312 m (1,024ft)	-10˚C - 19˚C	23-24/06 +26/06	Eggs uncommon, larvae rare and small, no pupae.
Vilhelmina	64˚37'N 16˚39'E	347 m (1147 ft)	-23˚C - 18˚C	25/06	Eggs uncommon, no larvae, no pupae.
Stockholm	59˚19'N 18˚4'E	52 m (171 ft)	-5˚C - 23˚C	24/07	Newly emerged adults abundant.
Östersund	63˚11'N 14˚40'E	312 m (1,024ft)	-10˚C - 19˚C	25/07 +27/07	Most adults newly emerged. Eggs, larvae and pupae present.
Vilhelmina	64˚37'N 16˚39'E	347 m (1147 ft)	-23˚C - 18˚C	26/07	Most adults newly emerged. Eggs, larvae and pupae present.
Malmö	55˚35'N 13˚02'E	73 m (240 ft)	-3˚C - 22˚C	20/09	No larvae or pupae. Adults mostly mature.
Stockholm	59˚19'N 18˚4'E	52 m (171 ft)	-5˚C - 23˚C	18/09	Some mature adults though the majority were newly emerged. Some pupae and some fourth instar larvae.
Östersund	63˚11'N 14˚40'E	312 m (1,024ft)	-10˚C - 19˚C	19/09	Late instar larvae and pupae very rare. The vast majority of adults were mature, few newly emerged.

Table A3.3: Phenological observation taken in 2002 at various points in Scandinavia (Tinsley, 2003).

Site	Latitude Longitude	Altitude	Average temperature	Date	Phenology observations
Malmö	55˚35'N 13˚02'E	73 m (240 ft)	-3˚C - 22˚C	06/06	Many fourth instar larvae. Almost all adults were overwintered. Pupae and newly emerged adults were very rare.
Stockholm	59˚19'N 18˚4'E	52 m (171 ft)	-5˚C - 23˚C	07/06	Many fourth instar larvae. Pre-pupae and pupae were common. Newly emerged adults were very rare.
Östersund	63˚11'N 14˚40'E	312 m (1,024ft)	-10˚C - 19˚C	08-09/06	Many fourth instar larvae and pre-pupae. Pupae were rare. No newly emerged adults collected. Some egg clutches.
Malmö	55˚35'N 13˚02'E	73 m (240 ft)	-3˚C - 22˚C	09/09	A significant proportion of adults were lightly pigmented and relatively young but none was recently emerged. No pupae or larvae present.
Stockholm	59˚19'N 18˚4'E	52 m (171 ft)	-5˚C - 23˚C	10/09	Late instar larvae not uncommon. A significant number of adults were young and a very small number newly emerged.
Ljusdal	61˚50'N 16˚05'E	145 m (476 ft)	-10˚C - 21˚C	12-15/09	A significant number of adults were young and a very small number newly emerged. A very few larvae.
Östersund	63˚11'N 14˚40'E	312 m (1,024 ft)	-10˚C - 19˚C	11/09	Adults of mixed age, some young. No pupae, a very small number of larvae.

Table A3.4: Phenological observations taken in 2011 at various points in Sweden.

Place	Longitude Latitude	Altitude	Average temperature	Observation date	Mite present	# Overwintered adult cohort collected	# New adult cohort collected	Other ladybird stages present
Vilhelmina	64˚37'N 16˚39'E	347 m (1147 ft)	-23˚C - 18˚C	03/07	No	28	0	No eggs. No larvae. No pupae.
Östersund	63˚11'N 14˚40'E	312 m (1,024 ft)	-10˚C - 19˚C	04/07	No	51	0	No eggs. No larvae. No pupae.
Ljusdal	61˚50'N 16˚05'E	145 m (476 ft)	-10˚C - 21˚C	17/06	No	88	0	No eggs, no larvae and no pupae.
				28/07		27	73	Some eggs, many third and fourth instar larvae. Pupae common.
Gävle	60˚40'N 17˚10'E	68 m (224 ft)	-7˚C - 21˚C	04/06	Yes	199	0	No eggs or larvae. No pupae.
				08/07		121	13	Third and fourth instar larvae are common. Pupae common.
Stockholm	59˚19'N 18˚4'E	52 m (171 ft)	-5˚C - 23˚C	21-26/05	Yes	366	0	Eggs common. Larvae were very rare.
				15-16/06		298	0	Eggs uncommon. First, second, third and fourth instar larvae are common. Pupae common.
				26/-27/07		286	199	No eggs. No larvae. No pupae.
Nässjö	57˚39'N 14˚41'E	375 m (1230 ft)	-7˚C - 21˚C	12/06	No	108	0	No eggs, no larvae and no pupae.
				08/07		29	76	Some eggs, Third and fourth instar larvae and pupae common.
Malmö	55˚35'N 13˚02'E	73 m (240 ft)	-3˚C - 22˚C	11/06	Yes	200	0	First, second, third and fourth instar larvae are common. Only few pupae.

Table A3.5: Phenological observations taken in 2013 at various points in Sweden.

Place	Longitude Latitude	Altitude	Average temperature	Observation date	Mite present	# Overwintered adult cohort collected	# New adult cohort collected	Other ladybird stages present
Östersund	63˚11'N 14˚40'E	312 m (1,024ft)	-10˚C - 19˚C	14/08	No	9	0	No eggs. Some fourth instar larvae. Some pupae.
Stockholm	59˚19'N 18˚4'E	52 m (171 ft)	-5˚C - 23˚C	21-24/05	Yes	174	0	No eggs. No larvae. No pupae.

References

Abbot, P. & Dill, L. M. (2001). Sexually transmitted parasites and sexual selection in the milkweed leaf beetle, *Labidomera clivicollis*. *Oikos*, **92:** 91-100.

Anbutsu, H. Shunsuke, G. & Fukatsu, T. (2008). High and low temperatures differently affect infection density and vertical transmission of male-killing *Spiroplasma* symbionts in *Drosophila* hosts. *Appl. Environ. Microbiol.*, **74 (19):** 6053-6059.

Apari, P., de Sousa, J. D. & Müller, V. (2014). Why sexually transmitted infections tend to cause infertility: an evolutionary hypothesis. *PLoS Pathog*., **10 (8)**: e1004111.

Ashby, B. & Gupta, S. (2013). Sexually transmitted infections in polygamous mating systems. *Phil. Trans. R. Soc. B.,* **368** (1613).

Ashraf, M. (2010). A study on laboratory rearing of ladybird beetle (*Coccinella septempunctata*) to observe its fecundity and longevity on natural and artificial diets. *International Journal of Biology,* **2 (1):** 165-174.

Berec, L. & Maxin, D. (2014). Why have parasites promoting mating success been observed so rarely? *Journal of Theoretical Biology,* **347:** 47-61.

Berriman, M., Haas, B. J., LoVerde, P. T., Wilson, R. A., Dillon, G. P., Cerqueira, G. C., *et al.* (2009). The genome of the blood fluke *Schistosoma mansoni*. *Nature*, **460:** 352-360.

Boots, M. & Knell, R. J. (2002). The evolution of risky behaviour in the presence of a sexually transmitted disease. *Proc. Biol. Sci.*, **269 (1491):** 585-589.

Borgia, G. (1986). Satin bowerbird parasites: a test of the bright male hypothesis. *Behav. Ecol. Sociobiol.*, **19:** 355-358.

Braig, H. R., Zhou, W., Dobson, S. & O'Neill, S. L. (1998). Cloning and characterization of gene encoding the major surface protein of the bacterial endosymbiont *Wolbachia. J. Bacteriol.,* **180 (9):** 2373-2383.

Brakefield, P. M. (1984a). Ecological studies on the polymorphic ladybird *Adalia bipunctata* in the Netherlands. I. Population biology and geographical variation of melanism. *The Journal of Animal Ecology*, **53 (3):** 761-774.

Brakefield, P. M. (1984b). Ecological studies on the polymorphic ladybird *Adalia bipunctata* in the Netherlands. II. Population dynamics, differential timing of reproduction and thermal melanism. *The Journal of Animal Ecology*, **53 (3):** 775-790.

Brakefield, P. M. & Willmer, P. G. (1985). The basis of thermal melanism in the ladybird *Adalia bipunctata*: Differences in reflectance and thermal properties between morphs. *Heredity*, **54:** 9-14.

Brockhurst, M. A., Chapman, T., King, K. C., Mank, J. E., Paterson, S. & Hurst, G. D. D. (2014). Running with the Red Queen: the role of biotic conflicts in evolution. *Proc. R. Soc. B.*, **281:** 20141382.

Brown, P. M. J., Adriaens, T., Bathon, H., Cuppen, J., Goldarazena, A., Hägg, T., Kenis, M., Klausnitzer, B. E. M., Kovář, I., Loomans, A. J. M., Majerus, M. E. N., Nedved, O., Pedersen, J., Rabitsch, W., Roy, H. E., Ternois, V., Zakharov, I. A. & Roy, D. B. (2008) *Harmonia axyridis* in Europe: spread and distribution of non-native coccinellid. *BioControl*, **53:** 5-21.

Buchner, P. (1966). Endosymbiosis of animals with plant microorganisms. *London Wiley.*

Chang, K. S., Shiraishi, T., Nakasuji, F. & Morimoto, N. (1991). Abnormal sex-ratio condition in the Walnut Leaf Beetle, *Gastrolina depressa* (Coleoptera: Chrysomelidae). *Applied Entomology and Zoology*, **26 (3):** 299-306.

Charlat, S., Hornett, E. A., Dyson, E. A., Ho, P. Y., Thi Loc, N., Schilthuizen, M., Davis, N., Roderick, G. K. & Hurst, G. D. D. (2005). Prevalence and penetrance variation of male-killing *Wolbachia* across Indo-Pacific populations of the butterfly, *Hypolimnas bolina*. *Mol. Ecol.*, **14 (11):** 3525-3530.

Charlat, S, Hornett, E. A., Fullard, J. H., Davies, N., Roderick, G., Wedell, N. & Hurst, G. D. D. (2007). Extraordinary flux in sex ratio. *Science,* **317 (5835):** 214.

Clopper, C. J. & Pearson, E. S. (1934). The use of confidence of fiducial limits illustrated in the case of the binomial. *Biometrika,* **26**: 404-413.

Colman, R. J., Beasley, T. M., Kemnitz, J. W., Johnson, S. C., Weindruch, A. & Anderson, R. M. (2014). Caloric restriction reduces age-related and all-cause mortality in rhesus monkey. *Nature Communications*, **5 (3557)**.

Convey, P. (1996). Overwintering strategies of terrestrial invertebrates in Antarctica – the significance of flexibility in extremely seasonal environments. *Eur. J. Entomol.*, **93:** 489-505.

Dorny, P. & Praet, N. (2007). *Taenia saginata* in Europe. *Veterinary Parasitology*, **149:** 22-24.

Douglas, A. E. (1998). Nutritional interactions in insect-microbial symbioses: Aphids and their symbiotic bacteria *Buchnera*. *Annual Review of Entomology*, **43:** 17-37.

Douglas, A. E. (2010). The symbiotic habit. *Princeton University Press*.

Elnagdy, S., Majerus, M. E. N., Gardener, M. Lawson Handley, L.-J. (2013). He different effects of male killer infection on fitness of ladybird hosts. *Journal of Evolutionary Biology*, **26 (8):** 1818-1825.

Ewald, P. W. (1983). Host-parasite relations, vectors and the evolution of disease severity. Annu. Rev. Ecol. Syst., 14: 465-485.

Ferrari, J., Darby, A. C., Daniell, T. J., Godfray, H. C. J. & Douglas, A. E. (2004). Linking the bacterial community in pea aphids with host-plant use and natural enemy resistance. *Ecol. Entomol.*, **29:** 60-65.

Ferrari, J. & Vavre, F. (2011). Bacterial symbionts in insects or the story of communities affecting communities. *Phil. Trans. R. Soc. B.*, **366:** 1389-1400.

Fukatsu, T., Tsuchida, T., Nikoh, N. & Koga, R. (2001). *Spiroplasma* symbiont of the pea aphid *Acyrthosiphon pisum* (Insecta: Homoptera). *Appl. Environ. Microbiol.*, **67 (3):** 1284-1291.

Fytrou, A., Schofield, P. G., Kraaijeveld, A. R. & Hubbard, S. F. (2006). *Wolbachia* infection suppresses both host defence and parasitoid counter-defence. *Proc. R. Soc. B.,* **273:** 791-796.

Gardner, M. J., Hall, N., Fung, E., White, O., Berriman, M, Hyman, R. W., *et al.* (2002). Genome sequence of the human malaria parasite *Plasmodium falciparum*. *Nature*, **419:** 498-511.

Goodacre, S. L., Martin, O. Y., Bonte, D., Hutchings, L., Woolley, C., Ibrahim, K., Thomas, C. F. G. & Hewitt, G. M. (2009). Microbial modification of host long-distance dispersal capacity. *BMC Biology*, **7:** 32.

Goodhead, I., Capewell, P., Bailey, J. W., Beament, T., Chance, M., Kay, S., Forrester, S., MacLeod, N., Taylor, M., Noyes, H. & Hall, N. (2013). Whole-genome sequencing of *Trypanosoma brucei* reveals introgression between subspecies that is associated with virulence. *mBIO*, **4 (4):** e00197.

Graham, R. I., Grzywacz, D., Mushobozi, W. L. & Wilson, K. (2012). *Wolbachia* in a major African crop pest increases susceptibility to viral disease rather than protects. *Ecology Letters*, **15 (9):** 993-1000.

Grossman, C. J. (1985). Interactions between the gondal steroids and the immune system. *Science*, **227:** 257-261.

Haddrill, P. R., Shuker, D. M. Mayes, S. & Majerus, M. E. N. (2007). Temporal effects of multiple mating on components of fitness in the two-spot ladybird, *Adalia* bipunctata (Coleoptera: Coccinellidae). *Eur. J. Entomol.,* **104 (1):** 393-398.

Haddrill, P. R., Shuker, D. M., Amos, W., Majerus, M. E. N. & Mayes, S. (2008). Female multiple mating in wild and laboratory populations of the two-spot ladybird *Adalia bipunctata*. *Mol. Ecol.*, **17:** 3189-3197.

Haddrill, P. R., Majerus, M. E. N. & Shuker, D. M. (2013). Variation in male and female mating behaviour among different populations of the two-spot ladybird, *Adalia* bipunctata (Coleoptera: Coccinellidae). *Eur. J. Entomol.,* **110 (1):** 87-93.

Haine, E. R. (2008). Symbiont-mediated protection. *Proc. R. Soc. B.*, **275:** 353-361.

Hart, B. L., Korinek, E. & Brennan, P. (1987). Post-copulatory genital grooming in male rats: prevention of sexually transmitted infections. *Physiol. Behav.*, **41:** 321-325.

Hedges, L. M., Brownlie, J. C., O'Neill, S. L. & Johnson, K. N. (2008). *Wolbachia* and virus protection in insects. *Science*, **322:** 702.

Hethcote, H. W. & Yorke, J. A. (1986). Gonorrhoea: transmission dynamics and control. *Biometrical Journal,* **28 (3)**: 304.

Hodek, I., Emden, H. F. van & Honek, A. (2012). Ecology and behaviour of the ladybird beetles (Coccinellidae). *Blackwell Publishing Ltd.*

Hornett, E. A., Charlat, S., Duplouy, A. M. R., Davies, N., Roderick, G. K., Wedell, N. & Hurst, G. D. D. (2006). Evolution of male-killer suppression in a natural population. *PLoS Biology*, **4 (9):** 1643-1648.

Huger, A. M., Skinner, S. W. & Werren, J. H. (1985). Bacterial infections associated with the son-killer trait in the parasitoid wasp *Nasonia* (=Marmoniella) *vitripennis* (Hymenoptera: Pteromalidae). *J. Invertebr. Pathol.*, **46 (3):** 272-280.

Hurst, L. D. (1991). The incidence and evolution of cytoplasmic male-killers. *Proc. R. Soc. Lond. B.*, **244:** 91-99.

Hurst, G. D. D., Majerus, M. E. N. & Walker, L. E. (1992). Cytoplasmic male killing elements in *Adalia bipunctata* (Linnaeus) Coleoptera: Coccinellidae) *Heredity*, **69:** 84-91.

Hurst, G. D. D., Majerus, M. E. N. & Walker, L. E. (1993a). The importance of cytoplasmic male killing elements in natural populations of the two spot ladybirds, *Adalia bipunctata* (Linnaeus) (Coleoptera: Coccinellidae). *Bio. J. Linn. Soc.*, **49:** 195-202.

Hurst, G. D. D. & Majerus, M. E. N. (1993b). Why do maternally inherited microorganisms kill males? *Heredity,* **71:** 81–95.

Hurst, G. D. D., Sharpe, R. G., Broomfield, A., Walker, L. E., Majerus, T. M. O., Zakharov, I. A. & Majerus, M. E. N. (1995). Sexually transmitted disease in a promiscuous insect, *Adalia bipunctata*. *Ecol. Entomol.*, **20:** 230-236.

Hurst, G. D. D., Hurst, L. D., & Majerus, M. E. N. (1997). Cytoplasmic sex ratio distorters, Pages 125-154 *in* S. L. O'Neill, A. A. Hoffmann, and J. H. Werren, eds. Influential passengers: microbes and invertebrate reproduction. *Oxford, U.K., O.U.P*.

Hurst, G. D. D., Schulenburg, G. V. D., Majerus, T. M. O., Bertrand, D. Zakharov, I. A., Baungaard, J., Völki, W., Stouthamer, R. & Majerus, M. E. N. (1999a). Invasion of one insect species, *Adalia bipunctata*, by two different male-killing bacteria. *Insect Molecular Biology*, **8 (1):** 133-139.

Hurst, G. D. D., Jiggins, F. M., van der Schulenburg, J. H., Bertrand, D., West, S. A., Goriacheva, I. I., Zakharov, I. A., Werren, J. H., Stouthamer, R. & Majerus, M. E. N. (1999b). Male-killing *Wolbachia* in two species of insects. *Proc. R. Soc. Lond. B.*, **266:** 735-740.

Hurst, G. D. D. & Jiggins, F. M. (2000). Male-killing bacteria in insects: mechanisms, incidence, and implications. *Emerg. Infect. Dis.,* **6 (4):** 329–336.

Hurst, G. D. D. & Frost, C. L. (2015). Reproductive parasitism: maternally inherited symbionts in a biparental world. *Cold Spring Harbor Laboratory Press*.

Husband, R. W. (1981). The African species of *Coccipolipus* with a description of all stages of *Coccipolipus solanophilae* (Acarina: Podapolipidae). *Rev. Zool. Afr.*, **95:** 283-299.

Ilinsky, Y. 2013. Coevolution of *Drosophila melanogaster* mtDNA and *Wolbachia* genotypes. PLoS One, 8(1): e54373. doi:10.1371/journal.pone.0054373**.**

Jaenike, J., Stahlhut, J., Boelio, L. M. & Unckless, R. L. (2010a). Association between *Wolbachia* and *Spiroplasma* within *Drosophila neotestacea*: an emerging symbiotic mutualism? *Molecular Ecology*, **19 (2):** 414-425.

Jaenike, J., Unckless, R., Cockburn, S. N., Boelio, L. M. & Perlman, S. J. (2010b). Adaptation via symbiosis: recent spread of a *Drosophila* defensive symbiont. *Science,* **329:** 212-215.

Jiggins, F. M., Hurst, G. D. D. & Majerus, M. E. N. (2000). Sex-ratio-distorting *Wolbachia* causes sex-role reversal in its butterfly host. *Proc. R. Soc. Lond. B,* **267:** 69-73.

Jiggins, F. M., Hurst, G. D. D., Schulenburg, J. H. G. vd. & Majerus, M. E. N. (2001). Two male-killing *Wolbachia* strains coexist within a population of the butterfly *Acraea encedon*. *Heredity*, **86:** 161-166.

Jiggins, F. M. & Tinsley, M. C. (2005). An ancient mitochondrial polymorphism in *Adalia bipunctata* linked to a sex-ratio-distorting bacterium. *Genetics*, **171 (3):** 1115-1124.

Kamada, N., Seo, S.-U., Chen, G. Y. & Nunez, G. (2013). Role of gut microbiota in immunity and inflammatory disease. *Nat. Rev. Immunol.* **13:** 321-335.

Kellner, R. L. L. (2002). Molecular identification of an endosymbiotic bacterium associated with pederin biosynthesis in *Paederus sabaeus*. *Insect Biochemistry and Molecular Biology*, **32:** 389-395.

Knell, R. J. (1998). Generation cycles. *Trends in Ecology and Evolution*, **13:** 186-190.

Knell, R. J. (1999). Sexually transmitted disease and parasite mediated sexual selection. *Evolution*, **56**: 1091-1100.

Knell, R. J. (2004). Syphilis in renaissance Europe: rapid evolution of an introduced sexually transmitted disease? *Proc. Biol. Sci.*, **271 (Suppl. 4):** 174-176.
Knell, R. J. & Webberley, K. M. (2004). Sexually transmitted diseases of insects: distribution, evolution, ecology and host behaviour. *Biol. Rev.,* **79:** 557-581.

Koch, R. L. (2003). The multi-coloured Asian lady beetle, *Harmonia axyridis*: a review of its biology, uses in biological control, and non-target impacts. *Journal of Insect Science*, **3:** 16-22.

Koch, H. & Schmid-Hempel, P. (2011). Socially transmitted gut microbiota protect bumble bees against an intestinal parasite. *Proc. Natl. Acad. Sci. USA*, **108:** 19288-19292.

Koella, J. (2009). Insect infection and immunity. *Oxford University Press,* 159-172.

Kokko, H., Ranta, E., Ruxton, G. & Lundberg, P. (2002). Sexually transmitted disease and the evolution of mating systems. *Evolution*, **56 (6):** 1091-1100.

Lakowski, B. & Hekimi, S. (1998). The genetics of caloric restriction in *Caenorhabditis elegans*. *PNAS,* **95 (22):** 13091-13096.

Lion, S., Baalen, M. van & Wilson, W. G. (2006). The evolution of parasite manipulation of host dispersal. *Proc. R. Soc. B.*, **273:** 1063-1071.

Lockhart, A. B., Thrall, P. H. & Antonovics, J. (1996). Sexually transmitted diseases in animals: ecological and evolutionary implications. *Biol. Rev.,* **71:** 415-471.

Loftus, B., Anderson, I., Davis, R. *et al.* (2005). The genome of the protist parasite *Entamoeba histolytica*. *Nature*, **433 (24):** 865-868.

Lus, Y. Y. (1947). Some rules of reproduction in populations of *Adalia bipunctata*. II. None-male strains in populations. *Dokl. Akad. Nauk. SSSR*, **57:** 951-954.

Madden, L. V., Nault, L. R., Heady, S. E. & Styer, W. E. (1984). Effect of maize stunting mollicutes on survival and fecundity of *Dalbulus* leafhopper vectors. *Ann. App. Biol.*, **105:** 431-441.

Majerus, M. E. N., O'Donald, P. & Weir, J. (1982). Evidence for preferential mating in *Adalia bipunctata*. *Heredity*, **49:** 37-49.

Majerus, M. E. N. (1994). Ladybirds. The New Naturalist. *Harper Collins Publishers*.

Majerus, T. M. O., Majerus, M. E. N., Knowles, B., Wheeler, J., Bertrand, D., Kuznetzov, V. N., Ueno, H. & Hurst, G. D. D. (1998). Extreme variation in the prevalence of inherited male-killing microorganisms between three populations of *Harmonia axyridis* (Coleoptera: Coccinellidae). *Heredity*, **81:** 683-691.

Majerus, M. E. N., Schulenburg, J. H. G. V. D. & Zakharov, I. A. (2000). Multiple cause of male-killing in a single sample of the spot ladybird, *Adalia bipunctata* (Coleoptera: Coccinellidae) from Moscow. *Heredity,* **84:** 605–609.

Majerus, T. M. O. & Majerus, M. E. N. (2012). Male-killing in the Coccinellidae: testing the predictions. *Evol. Ecol.*, **26:** 207-225.

Marples, N. M., Brakefield, P. M. & Cowie, R. J. (1989). Differences between the 7-spot and 2-spot ladybird beetles (Coccinellidae) in their toxic effects on a bird predator. *Ecol. Entomol.*, **14:** 79-84.

Marples, N. M., Veelen, van W. & Brakefield, P. M. (1994). The relative importance of colour, taste and smell in the protection of aposematic insect *Coccinella septempunctata*. *Anim. Behav.*, **48:** 967-974.

McCay, C. M., Crowell, M. F. & Maynard, L. A. (1935). The effect of retarded growth upon the length of life span and upon the ultimate body size. *The Journal of Nutrition*, **10:** 63-79.

Mendes, R., Kruijt, M., de Bruijn, I., Dekkers, E., van der Voort, M., Schneider, J. H. M., Piceno, Y. M., DeSantis, T. Z., Andersen, G. L., Bakker, P. A. H. M. & Raaijmakers, J. M. (2011). Deciphering the rhizoshpere microbiome for disease-suppressive bacteria. *Science*, **332:** 1097-1100.

Montenegro, H., Solferini, V. N., Klaczko, L. B. & Hurst, G. D. D. (2005). Male-killing *Spiroplasma* naturally infecting *Drosophila melanogaster*. *Insect Molecular Biology*, **14 (3):** 281-287.

Moore, S. L. & Wilson, K. (2002). Parasites as a viability cost of sexual selection in natural populations of mammals. *Science*, **297 (5589):** 2015-2018.

Morris, R.J., Lewis, O.T. & Godfray, H. Ch. J. (2004). Experimental evidence for apparent competition in a tropical forest food web. *Nature,* **428:** 310-313.

Nahrung, H. F. & Allen, G. R. (2004). Sexual selection under scramble competition: mate location and mate choice in the eucalypt leaf beetle *Chrysophtharta agricola* (Chapuis) in the field. *J. Insect Behav.*, **17:** 353-366.

Nahrung, H. F. & Clarke, A. R. (2007). Sexually-transmitted disease in a sub-tropical eucalypt beetle: infection of the fittest? *Evol. Ecol.*, **21:** 143-156.

Nakamura, Y., Kawai, S., Yukuhiro, F., Ito, S., Gotoh, T., Kisimoto, R., Yanase, T., Matsumoto, Y., Kageyama, D. & Noda, H. (2009). Prevalence of *Cardinium* bacteria in planthoppers and spider mites taxonomic revision of *'Candidatus Cardinium hertigii'* based on detection of a new *Cardinium* group from biting midges. *Appl. Environ. Microbiol.*, **75 (21):** 6757-6763.

Neelakanta, G., Sultana, H., Fish, D., Anderson, J. F. & Fikrig, E. (2010). *Anaplasma phagocytophilum* induces *Ixodes scapularis* ticks to express an antifreeze glycoprotein gene that enhances their survival in the cold. *J. Clin. Invest.*, **120 (9):** 3179-3190.

Nunn, C. L., Gittleman, J. L. & Antonovics, J. (2000). Promiscuity and the primate immune system. *Science*, **290:** 1168-1170.

Nunn, C. L. (2003). Behvioural defences against sexually transmitted diseases in primates. *Anim. Behav.*, **66:** 37-48.

Oliver, K. M., Russell, J. A., Moran, N. A. & Hunter, M. S. (2003). Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. *Proc. Natl. Acad. Sci. USA,* **100 (4):** 1803-7.

Oliver, K. M., Moran, N. A. & Hunter, M. S. (2005). Variation in resistance to parasitism in aphids is due to symbionts not host genotype. *PNAS*, **102 (36):** 12795-12800.

Omkar, & Pervez, A. (2005). Ecology of the two-spotted ladybird, *Adalia bipunctata*: a review. *J. Appl. Entomol.,* **129 (9-10):** 465-474.

Osaka, R., Nomura, M., Watada, M. & Kageyama, D. (2008). Negative effects of low temperatures on the vertical transmission and infection density of *Spiroplasma* endosymbiont in *Drosophila hydei*. *Curr. Microbiol.*, **57:** 335-339.

Perrot-Minnot, M.-J., Guo, L. R. & Werren, J. H. (1996). Single and double infections with *Wolbachia* in the parasitic wasp *Nasonia vitripennis*: effects on compatibility. *Genetic*, **143:** 961-972.

Perry, J. C. & Rowe, L. (2008a). Neither mating nor spermatophore feeding influences longevity in a ladybird beetle. *Ethology,* **114:** 504-511.

Perry, J. C. & Rowe, L. (2008b). Ingested spermatophores accelerate reproduction and increase mating resistance but are not a source of sexual conflict. *Animal Behaviour*, **76:** 993-1000.

Perry, J. C., Sharpe, D. M. T. & Rowe, L. (2009). Condition-dependent female remating resistance generates sexual selection on male size in a ladybird beetle. *Animal Behaviour,* **77 (3):** 743-748.

Perry, J. C. (2011). Mating stimulates female feeding: testing the implications for the evolution of nuptial gifts. *J. Evol. Biol.,* **24:** 1727-1736.

Perry, J. C. & Tse, C. T. (2013). Extreme costs of mating for male two-spot ladybird beetles. *Plos One,* **8 (12).**

Phoofolo, M. W. & Obrycki, J. J. (2000). Demographic analysis of reproduction in Nearctic and Palearctic populations of *Coccinella septempunctata* and *Propylea quatuordecimpunctata*. *BioControl* **45:** 25-43.

Poole, D. N. & McClelland, R. S. (2013). Global epidemiology of *Trichomonas vaginalis*. *Sex. Transm. Infect.*, **89:** 418-422.

Randall, K., Majerus, M. E. N. & Forge, H. (1992). Characteristic for sex determination in British ladybirds (Coleoptera: Coccinellidae). *Entomologist,* **111:** 109-122.

Randerson, J. P., Smith, N. G. C & Hurst, L. D. (2000). The evolutionary dynamics of male-killers and their host. *Heredity*, **80:** 152-160.

Rankin, D. J. & Kokko, H. (2007). Do males matter? The role of males in population dynamics. *OIKOS*, 116 (2): 335-348.

Rodriguez, R. J., Henson, J., Volkenburgh, E., Hoy, M., Wright, L., Beckwith, F., Kim, Y.-O. & Redman, R. S. (2008). Stress tolerance in plants via habitat-adapted symbiosis. *The ISME Journal*, **2:** 404-416.

Ryder, J. J., Webberley, K. M., Boots, M. & Knell, R. J. (2005). Measuring the transmission dynamics of a sexually transmitted disease. *PNAS*, **102 (42):** 15140-15143.

Ryder, J. J., Hathway, J. & Knell, R. J. (2007). Constraints on parasite fecundity and transmission in an insect-STI system. *Oikos*, **116:** 578-584.

Ryder, J. J., Pastok, D., Hoare, M.-J., Bottery, M., Boots, M., Knell, R. J., Atkinson, D., & Hurst, G. D. D. (2013). Spatial variation in food supply, mating behaviour, and sexually transmitted disease epidemics. *Behavioural Ecology.*

Ryder, J. J., Hoare, M.-J., Pastok, D., Bottery, M., Boots, M., Fenton, A., Atkinson, D., Knell, R. J., & Hurst, G. D. D. (2014). Disease epidemiology in arthropods is altered by the presence of non-protective symbionts. *The American Society of Naturalists*, **183 (3):** 89-104.

Saunders, D. S., Sutton, D. & Jarvis, R. A. (1970). The effect of host species on diapause induction in *Nasonia vitripennis*. *Journal of Insect Physiology*, **16 (3):** 405-416.

Scarborough, C. L., Ferrari, J. & Godfray, H. C. J. (2005). Aphid protected from pathogen by endosymbiont. *Science* **310:** 3781.

Scheridan, L. D., Poulin, R., Ward, D. F. & Zuk, M. (2000). Sex differences in parasitic infections among arthropod hosts: is there a male bias? *Oikos*, **88 (2):** 372-334.

Schmid-Hempel, P. & Sadd, B. M. (2009). Insect infection and immunity. *Oxford University Press,* 225-240.

Seeman, O. D. & Nahrung, H. F. (2004). Female-biased parasitism and the importance of host generation overlap in a sexually transmitted parasite in beetles. *Journal of Parasitology*, **90 (1):** 114-118.

Sprong, H., Cacciò, S. M., Giessen, van der J. W. B (on behalf of the ZOOPNET network and partners) (2009). Identification of zoonotic genotypes of *Giardia duodenalis*. *PLoS Negl. Trop. Dis.,* **3 (12):** e558.

Storey, K. B. & Storey, J. M. (1996). Natural freezing survival in animals. *Annu. Rev. Ecol. Syst.*, **27:** 365-386.

Summers, C. G., Newton, A. S., Jr. & Opgenorth, D. C. (2004). *Environ. Entomol*., **33 (6):** 1644-1651.

Tenter, A. M., Heckeroth, A. R. & Weiss, L. M. (2000). *Toxoplasma gondii*: from animals to human. *J. Parasitology*, **30:** 1217-1258.

Thrall, P. H., Antonovics, J. & Bever, J. D. (1997). Sexual transmission of disease and host mating systems: within-season reproductive success. *The American Naturalist,* **149 (3):** 485-506.

Thrall, P. H., Antonovics, J. & Dobson, A. P. (2000). Sexually transmitted diseases in polygynous mating systems: prevalence and impact on reproductive success. *Proc. R. Soc. Lond. B.,* **267:** 1555-1563.

Tinsley, M. C. (2003). The ecology and evolution of male-killing bacteria in ladybirds. PhD Thesis, University of Cambridge.

Waterman, J. M. (2010). The adaptive function of masturbation in a promiscuous African grey squirrel. *PLoS ONE*, **5:** 7.

Webberley, K. M. & Hurst, G. D. D. (2002). The effect of aggregative overwintering on an insect sexually transmitted parasite system. *Journal of Parasitology*, **88:** 707-712.

Webberley, K. M., Hurst, G. D. D., Buszko, J. & Majerus, M. E. N. (2002). Lack of parasite-mediated sexual selection in a ladybird/sexually transmitted disease system. *Animal Behaviour*, **63:** 131-141.

Webberley, K. M, Hurst, G. D. D., Husband, R., Schulenburg, G. V. D, Sloggett, J. J., Isham, V., Buszko, J. & Majerus, M. E. N. (2004). Host reproduction and a sexually transmitted disease: causes and consequences of *Coccipolipus hippodamiae* distribution on coccinellid beetles. *Journal of Animal Ecology*, **73:** 1-10.

Webberley, K. M., Buszko, J, Isham, V. & Hurst, G. D. D. (2006a). Sexually transmitted disease epidemics in a natural insect population. *Journal of Animal Ecology*, **75:** 33-43.

Webberley, K. M., Tinsley, M. C. Sloggett, J. J., Majerus, M. E. N. & Hurst, G. D. D. (2006b). Spatial variation in the incidence of a sexually transmitted parasite of the ladybird beetle *Adalia bipunctata* (Coleoptera: Coccinellidae). *Eur. J. Entomol.,* **103:** 793-797.

Welch, V. L., Sloggett, J. J., Webberley, K. M. & Hurst, G. D. D. (2001). Short-range clinal variation in the prevalence of a sexually transmitted fungus associated with urbanisation. *Ecol. Entomol.*, **26:** 547-550.

Werren, J. H., Hurst, G. D., Zhang, W., Breeuwer, J. A. J., Stouthamer, R. & Majerus, M. E. N. (1994). *Rickettsial* relative associated with male killing in the ladybird beetle (*Adalia bipunctata*). *J. Bacteriol.,* **176:** 388-394.

Werren, J. H., Guo, L. W., & Windsor, D. W. (1995). Distribution of *Wolbachia* in neotropical arthropods. *Proc. R. Soc. B.*, **262:** 197-204.

Woiwod, I. P. & Hanski, I. (1992). Patterns of density dependence in moths and aphids. *J. Anim. Ecol.*, **61 (3):** 619-629.

Xie, J., Vilchez, I. & Mateos, M. (2010). *Spiroplasma* bacteria enhance survival of *Drosophila hydei* attacked by the parasitic wasp *Leptopilina heterotoma*. *PLoS ONE* **5**, e12149.

Zakharov, I. A., Hurst, G. D. D., Chernysheva, N. E. & Majerus, M. E. N. (1996). Maternally inherited bacterium causing female bias in the St. Petersburg population of *Adalia bipunctata* does not belong to the genus *Rickettsia*. *Russian Journal of Genetics*, **32 (11):** 1303-1306.

Zakharov, I. A. & Shaikevich, E. V. (2001). The Stockholm populations of *Adalia bipunctata* (L) (Coleoptera: Coccinellidae) – a case of extreme female-biased population sex ratio. *Hereditas*, **134:** 263-266.

Zhou, W., Rousset, F. and O'Neil, S. (1998). Phylogeny and PCR–based classification of *Wolbachia* strains using wsp gene sequences. *Proc. R. Soc. Lond. B.,* 22, **265 (1395**): 509-515.